Kristin Gellein

High resolution inductively coupled plasma mass spectrometry: Some applications in biomedicine

Thesis for the degree philosophiae doctor

Trondheim, June 2008

Norwegian University of Science and Technology
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Trondheim, March 2008

Kristin Gellein

List of papers included

The thesis is based on the following five papers:

I
Gellein, K., Flaten, T.P., Erikson, K., Aschner, M. and Syversen, T. Leaching of trace elements from biological tissue by formalin fixation. *Biological Trace Element Research* 2008, 121 (3); 221-225

II
Gellein, K., Roos, P.M., Evje, L., Vesterberg, O., Flaten, T.P., Nordberg, M. and Syversen, T. Separation of proteins including metallothionein in cerebrospinal fluid by size exclusion HPLC and determination of trace elements by HR-ICP-MS. *Brain Research* 2007, 1174; 136-142

III
Gellein, K., Lierhagen, S., Brevik, P.S., Teigen, M., Kaur, P., Singh, T., Flaten, T.P. and Syversen, T. Trace element profiles in single strands of human hair determined by HR-ICP-MS. *Biological Trace Element Research* 2008 (accepted)

IV
Gellein, K., Skogholt, J.H., Aaseth, J., Thoresen, G.B., Lierhagen, S., Steinnes, E., Syversen T. and Flaten, T.P. Trace elements in cerebrospinal fluid and blood from patients with a rare progressive central and peripheral demyelinating disease. *Journal of the Neurological Sciences* 2008, 226; 70-78

V
Gellein, K., Syversen, T., Steinnes, E., Nilsen, T.I.L., Dahl, O.P., Mitrovic, S., Duraj, D. and Flaten, T.P. Trace elements in serum from patients with Parkinson's disease – a prospective case-control study. The Nord-Trøndelag Health Study (HUNT). *Brain Research* (Submitted)

Summary

Even though trace elements are present at minute amounts in the human body, they have a considerable impact on human health, either as essential elements in biochemical functions indispensable for life, or on the contrary, interfering with vital processes. Knowledge of the optimal concentrations of trace elements in the human body is therefore of great importance. Since the first systematic determinations of trace elements in human body fluids started in the 1940s there has been an incredible development in analytical instrumentation. The objective of this thesis is to demonstrate successful applications of HR-ICP-MS (high resolution inductively coupled plasma mass spectrometry) in biomedicine.

Research on trace elements in humans is challenging because of very low levels and many different types of matrices. The first important issue regarding trace element analysis is sampling and sample storage. It is essential to control all possible sources of contamination and other factors that can influence the concentrations. Preservation of biological samples is often required, and effects of the frequently used preservation and storage of biological tissue in formalin have been examined in this work. The concentrations of 20 trace elements were determined in formalin where brain samples had been stored at different time intervals ranging from few weeks to several years. The results show that storage of biological tissue in formalin may result in losses of trace elements from the tissue to the formalin, and that the leakage is time-dependent. This emphasizes the importance of controlling all steps from sample collection to analysis.

With its low detection limits, high resolution and multielement capability, HR-ICP-MS offers a considerable potential for further understanding the role of trace elements in biological material. These features were used to develop a method to study protein-bound metals in cerebrospinal fluid (CSF). CSF samples from eight healthy persons were separated by size exclusion HPLC and the resulting fractions were analyzed using HR-ICP-MS. The major challenge in this work was the very low concentrations as only 100 µl CSF was injected to the column resulting in 35 fractions of 0.75 ml. It was possible to determine more than 10 elements of clinical interest in the CSF fractions and the method provides an opportunity to study MT and other metal binding proteins in CSF.

Further, the potential to study exposure and intake of trace elements by HR-ICP-MS was explored by analyzing hair strands of five occupationally unexposed subjects. The trace element profiles of single hair strands were determined by analyzing 1 cm long segments. The challenge in this study

was again the extremely small sample size, as the samples had an average weight of 0.05 mg. It was possible however to obtain results for 12 elements in these minute samples and valuable information about intake and exposure for Hg, Se and Sr was obtained.

HR-ICP-MS has the potential to be an excellent tool for obtaining information about disease development and progress. A rare and relatively unexplored neurodegenerative disease (Skogholt's disease) was studied. The trace element concentrations in whole blood, plasma and CSF were determined in Skogholt patients, multiple sclerosis patients and controls. Increased levels of Cu, Fe, Zn, Se and S in CSF were found in CSF from Skogholt patients. These increased levels were not reflected in blood, and it is quite obvious that the increased levels are not caused by increased environmental exposure. The results suggest that the increased levels of these elements in CSF are due to a leakage of metal binding proteins from blood to the CSF.

Trace elements have been implicated in the development of Parkinson's disease (PD), and a study was performed on trace elements in serum from Parkinson patients collected in 1995-97, 4-12 years before they were diagnosed with the disease. New samples from more than half of these patients were collected in 2007. No significant differences were found between preclinical levels and controls, except for a lower level of Hg in the patient group. However, when trace element serum levels in patients from before and after they were diagnosed were compared, significant differences for several elements were found. This suggests that trace element imbalances found in PD patients may be a result of disease development rather than a causal factor.

HR-ICP-MS offers a considerable potential for further understanding the role of trace elements in humans. Biological material is often available for analysis only in small amounts. HR-ICP-MS gives the opportunity of simultaneous quantification of many trace elements even in very small samples and with very low detection limits. This promotes new research in the field of trace elements in biological material. HR-ICP-MS also reduces the time and cost per analysis and broadens the amount of information available from a single specimen.

Contents

ACKNOWLEDGEMENTS **1**

LIST OF PAPERS INCLUDED **3**

SUMMARY **5**

CONTENTS **7**

1 INTRODUCTION **9**

1.1 TRACE ELEMENTS IN HUMANS 9
1.1.1 Introduction *9*
1.1.2 Functions of trace elements *9*
1.1.3 Deficiency *10*
1.1.4 Toxicity *11*
1.1.5 Interactions *12*
1.1.6 Metalloproteins *12*
1.1.6 Trace elements and neurodegenerative disorders *13*
1.2 TRACE ELEMENT DETERMINATIONS IN BIOLOGICAL MATERIAL 14
1.2.1 Atomic absorption spectrometry *14*
1.2.2 Inductively coupled plasma atomic emission spectrometry *15*
1.2.3 Inductively coupled plasma mass spectrometry *15*
1.2.4 High Resolution ICP-MS *16*
1.2.5 Trace element speciation *17*
1.3 CHALLENGES IN SAMPLING AND ANALYSIS 17
1.3.1 Sample collection and contamination control *17*
1.3.2 Sample preparation *19*
1.4 QUALITY CONTROL 20
1.4.1 Precision and accuracy *20*
1.4.2 Reference materials *21*
1.4.3 Internal standard and matrix effects *21*
1.4.5 Memory effects *22*

2 SUMMARY OF THE INDIVIDUAL PAPERS **23**

2.1 PAPER I 23
2.2 PAPER II 23
2.3 PAPER III 24
2.4 PAPER IV 25
2.5 PAPER V 25

3 CONCLUSIONS **27**

4 REFERENCES **29**

PAPER I-V **35**

1 Introduction

1.1 Trace elements in humans

1.1.1 Introduction

Trace elements are often defined as those elements having concentrations less than 100 mg/g (0.01%) in the human body (Peereboom, 1985). Another definition of a trace element is all naturally occurring elements in the periodic table, except minor and major elements and noble gases (Lindh, 2005). The major elements (H, O, C and N) make up 96% of the human body, whereas the minor elements (Na, K, Ca, Mg, P, S and Cl) comprise 3.8% of the human body (Lindh, 2005). There are 90 naturally occurring elements in the Periodic Table, and by this definition, the trace elements include 73 elements. Of these 73, 18 elements are considered to be essential or possibly essential trace elements: Li, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, W, Mo, Si, Se, F, I, As, Br and Sn (Lindh, 2005). A definition of an essential element is given by Mertz (Mertz, 1981):
An element is essential when a deficient intake consistently results in an impairment of a function from optimal to suboptimal and when supplementation with physiological levels of this element, but no other, prevents or cures this impairment.
Essential trace elements are required at optimum levels for a number of metabolic and physiological processes in the human body typically in amounts ranging from 50 μg to 18 mg per day (Mertz, 1981). However, excess concentrations of both essential and non-essential metals can be toxic, while insufficient levels may lead to metabolic failure.

1.1.2 Functions of trace elements

Trace elements have many important functions in the human body. The most important functions are as co-factors in essential enzymes and proteins, stabilizers in membranes (e.g. Zn in biomembranes), elements of structures, and essentiality for hormonal function (e.g. iodine in thyroid hormones) (Peereboom, 1985). Zinc, for example, is known to be an essential co-factor in at least 100 enzymes and is important for DNA transcription and regulation (Peereboom, 1985).

Essential elements are subject to homeostatic control mechanisms that may include absorption, excretion as well as tissue retention. It is important to ensure a safe and optimum supply of essential trace elements for the

performance of essential functions. Any essential element becomes toxic when it enters an organism in large amounts. On the other hand, inadequate intake may impair cellular and physiological function and often causes illness. Fig. 1 illustrates the relation between a biological function and the concentration in the body or dietary intake of a trace element. The increase is followed by a plateau representing the maintenance of optimal function through homeostatic regulation, and a decline of the function toward zero as the regulatory mechanisms are overcome by increasing concentrations that become toxic (Mertz, 1981). Each element has its own specific curve, which differs from that of other trace elements (for example the width of the plateau), but the model is probably applicable to most essential trace elements. Two conclusions from this model are important: 1) for each element there is a range of safe and adequate exposures, within which homeostasis is able to maintain optimal tissue concentrations and function; 2) every trace element is potentially toxic when the range of adequate and safe exposure is exceeded (Mertz, 1981).

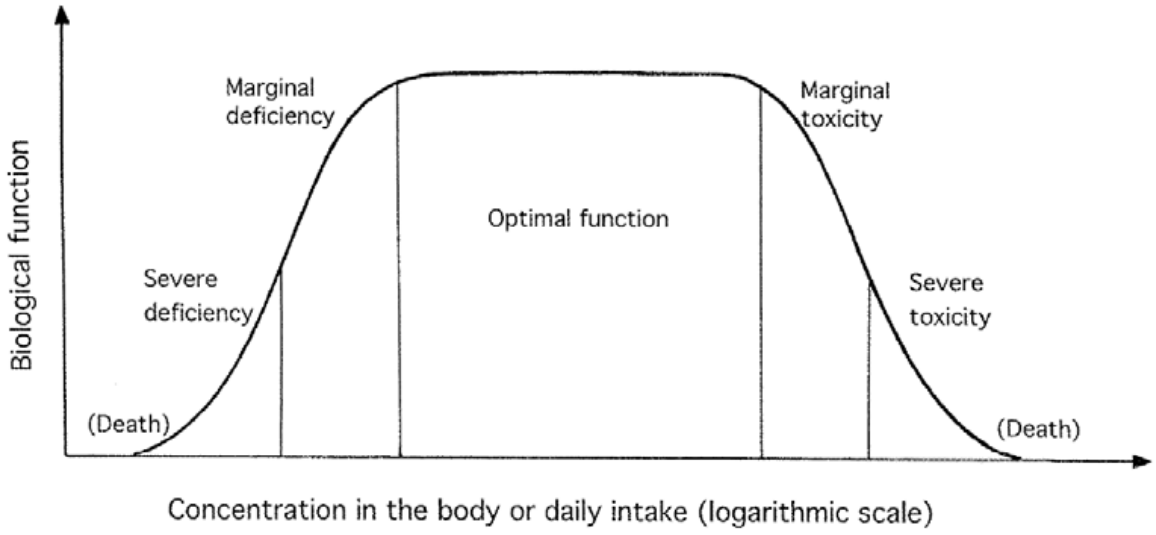

Figure 1. Dose-response curve for essential trace elements (Mertz, 1981). The figure is reproduced after Flaten (1997).

1.1.3 Deficiency

Deficiency of trace elements may be caused by inadequate nutrition. The world's most prevalent nutritional disorder is iron deficiency (Menkes, 1997). Iodine deficiency is also a widespread problem, affecting hundreds of millions of people worldwide (Hetzel, 2005). In fact, iodine is probably the first trace element to be associated with a human disease and salts of iodine was used therapeutically as early as in 1820 (Smith, 1987). Iodized salt have

proven to be very effective in preventing iodide deficiency disorders all over the world and has substantially reduced goitre caused by iodine deficiency. Deficiencies can also originate from genetic or inherited disorders in trace element metabolism. Inherited neurological disorders in which metals are involved in the pathogenesis have been described, e.g. Menke's disease and acrodermatitis enteropathica. Menkes' disease is a genetic copper deficiency disorder were copper transport is disrupted (Menkes, 1999). The disease is usually fatal in early childhood. Acrodermatitis enteropathica is a rare autosomal recessive disorder where a decrease in Zn absorption leads to Zn deficiency (Krogh and Syversen, 1975).

1.1.4 Toxicity

Paracelsus (1493-1541) stated that everything is toxic; it is just a matter of dose. Essential elements can also be toxic if excessive amounts are ingested. Manganese, for example, is an essential trace element in the human diet, but occupational exposure (e.g. mine-workers) shows that such inhalation can trigger psychotic behaviour and Parkinson-like symptoms, also called manganism (Mena and Marin, 1967). Arsenic poisoning is a problem of increasing recognition. Due to contamination by natural geological occurrence, arsenic is present in high amounts in drinking water in Bangladesh and other Asian countries, causing poisoning of the population (Mukherjee et al., 2006). Some elements such as cadmium, lead and mercury are classically catalogued as toxic elements since they have deleterious effects even at very low levels (Versieck and Cornelis, 1989). There are many examples of severe poisoning by these elements. Pb is neurotoxic at very low concentrations (Skerfving and Bergdahl, 2007), in fact during the last 2-3 decades the blood lead level considered safe has decreased from more than 20 to less than 10 μg/dl. Pb has even been implicated in the downfall of the Roman Empire, as the ruling classes had lead piping installed in their homes and drank from goblets containing lead (Nriagu, 1983). Mercury is also highly neurotoxic and the world's largest environmental mercury poisoning was discovered in Minamata, Japan, in 1956. Severe poisoning with many fatal cases and prenatal poisonings occurred and the source of poisoning was fish and shellfish heavily contaminated by industrial discharge of mercury into the local waters (Harada, 1995). In Iraq, several hundred farmers died after eating seed grain dressed with methylmercury (Bakir et al., 1973).

1.1.5 Interactions

The effects of deficiencies and toxicity of trace elements do not solely depend on the levels at which they are present, but also on a number of other factors (Becking et al., 2007):

- the chemical form,
- food source or dietary matrixes,
- age,
- gender,
- nutritional state,
- interaction with other elements.

Speciation, which means the specific form in which an trace element is present in a certain matrix, is of outmost importance. As an example, water-soluble As salts are carcinogenic to man, whereas As bound to proteins (organic) have a low toxicity (Peereboom, 1985). Many toxic and essential trace elements are interrelated in some way, mainly because of similar chemical characteristics. This may cause biological effects, as there is a lack of complete specificity of mechanisms involved in absorption, transport and bio-interaction of the individual metals. Such interactions can both enhance adverse effects and protect against toxicity. In general, a deficiency of essential elements may increase toxicity of heavy metals, while an excess in many situations is protective. For example, a deficiency of Ca, Fe or Zn, enhances susceptibility of Cd and Pb toxicity (WHO, 1996), while selenium protects against mercury toxicity by binding the mercury in large proteins (Flaten, 1997). A classical example of element-element interactions is that a high intake of Mo may induce Cu deficiency in ruminants (Mills and Davis, 1987).

1.1.6 Metalloproteins

One-third of all proteins are metalloproteins, chemical combinations of protein atoms (carbon, nitrogen, oxygen, hydrogen, sulphur) with ions of metals such as iron, calcium, copper, and zinc. Metalloproteins have important roles in the availability and uptake of trace elements as well as in transport and storage. The haemoglobin, for example, that carries oxygen in the bloodstream, is an iron-containing metalloprotein (Morris, 1987). The metal ions in metalloproteins are critical to the protein's function, structure, or stability. In fact, numerous essential biological functions require metal ions, and most of these metal ion functions involve metalloproteins. Metalloproteins are involved in electron transport, oxygen storage, metal

transport, chemical bond hydrolysis, redox processes, and synthesis of biological compounds (Lindh, 2005).

An important sub-group of metalloproteins is metallothioneins (MT). Metallothioneins are low molecular weight intracellular proteins with a unique capacity to bind 7 metal ions through 20 sulphydryl groups (Nordberg, 1998). High concentrations of the protein are found in liver, kidney, intestine and pancreas. It is thought to be involved in the regulation of copper and zinc metabolism, and in the detoxification of heavy metals (Nordberg and Cherian, 2005). Four major forms of MT have been described: MT-1, MT-2, MT-3 and MT-4 (Nordberg et al., 1971). MT is an inducible protein, and its synthesis can be induced in response to many substances, including zinc, cadmium, copper, mercury and some non-metallic compounds. Abnormalities in MT have been reported in multiple neurological disorders (Aschner and West, 2005) and it is possible that MTs may have important roles in several human neurological diseases such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (Johansson et al., 2005; Hozumi et al., 2004; Roos et al., 2006; Barany et al., 2002; Forte et al., 2004).

1.1.6 Trace elements and neurodegenerative disorders

The pathogenesis and aetiology of neurodegenerative disorders such as Parkinson's disease, amyotrophic lateral sclerosis, and Alzheimer's disease are still unknown. Trace elements have been implicated in the pathogenesis and/or aetiology of several neurodegenerative diseases (Sayre et al., 2000; Zatta et al., 2003). The redox-active transition metals Cu and Fe seem to be involved in mediating the processes leading to oxidative stress. Considerable evidence implicates crucial roles of oxidative stress in the pathogenesis of major neurodegenerative disorders (Halliwell, 2006). Mn is a neurotoxic trace element and overexposure of Mn can cause manganism, a neurological syndrome that resembles Parkinson's disease (Mena and Marin, 1967; Dobson et al., 2004). Accumulation of Al in the brain is often found in Alzheimer type neurofibrillary degeneration and Al has therefore been suspected to be involved in the pathogenesis of Alzheimer's disease (Crapper et al., 1973; Flaten, 2001).

1.2 Trace element determinations in biological material

The determination of trace elements in biological fluids and tissues serves several purposes, including quantification of essential trace elements in normal and disease condition, detection of potentially toxic metals, and diagnosis of trace element deficiency and trace element related diseases (e.g. (Heitland and Köster, 2006; Schütz et al., 2005; McDiarmid et al., 2006; Carrizales et al., 2006; Chen et al., 2007; Forte et al., 2005; Rodushkin et al., 2000; Kassu et al., 2005). The accurate determination of environmentally and occupationally relevant trace elements can be an important tool in the assessment of exposure, diagnosis and treatment of adverse health effects (Gellein et al., 2008b).

Biological materials are difficult matrices for trace element analysis because they contain large amounts of proteins, inorganic salts and small organic molecules, which may cause serious matrix effects (Marchante-Gayón, 2004). However, advances in instrumentation over the past 1-2 decades have led to extraordinary improvements in the precision and sensitivity of trace element analysis. The most common techniques used for trace element determination in human biological material are flame atomic absorption spectrometry (flame AAS), graphite furnace atomic spectrometry (graphite AAS), inductively coupled plasma atomic emission spectrometry (ICP-AES) and inductively coupled plasma mass spectrometry (ICP-MS).

1.2.1 Atomic absorption spectrometry

Atomic absorption spectrometry (AAS) has frequently been used for the determination of trace elements in a wide range of materials. The methods measure the amount of energy (in the form of photons of light) absorbed by the sample. Ultraviolet and visible atomic spectra are obtained by converting the components of a sample into atoms or elementary ions by suitable heat treatment (Slavin, 1994). The light source is usually a hollow cathode lamp, which contains the same element as that to be determined. The precision and accuracy of atomic methods are critically dependent upon the atomization step. Atomization of the compound in solution can be achieved either by introducing the sample into a flame (flame AAS), or by introducing the sample into a graphite tube where it is heated electrically to convert the sample into a cloud of free atoms (graphite AAS).

Table 1. Flame AAS

Advantages	Drawbacks
- simple and easy - low costs - high reproducibility - high precision	- high detection limit - sample volume (several ml) - traditionally a single element technique

Table 2. Graphite AAS

Advantages	Drawbacks
- relatively low cost - small sample volume (10-100µl) - low detection limit	- slow (>2 min pr. sample) - traditionally a single element technique

1.2.2 Inductively coupled plasma atomic emission spectrometry

Inductively coupled plasma is an electrical conducting gaseous mixture of argon, argon ions and electrons. The plasma is formed from a stream of argon gas, which is energized by a high-energy, radio-frequency field (Montaser et al., 1998). This leads to high atomization and ionization of the flowing argon and an excitation temperature of 5500-8000°K is generated (Montaser et al., 1998). The sample is introduced into the plasma as an aerosol and the high temperature causes a very efficient desolvation, volatilization, atomization, excitation and ionization of the sample (Montaser et al., 1998). ICP-AES is a type of emission spectrometry that uses the ICP to produce excited atoms that emit electromagnetic radiation at a wavelength characteristic of a particular element. The intensity of this emission is used to determine the concentration of the element within the sample.

Table 3. ICP-AES

Features	Drawbacks
- multielement capacity - high precision	- high detection limits

1.2.3 Inductively coupled plasma mass spectrometry

The very high temperature of the argon plasma and its effectiveness as an ionization source were further applied in the development of ICP-MS in 1980 (Houk et al., 1980). The ICP-MS instrument combines the plasma as ion source with a mass spectrometer. Quadropole (Q) mass analyzers have been the most popular mass filters in ICP-MS owing to their relatively low

costs and easy handling (Marchante-Gayón et al., 1999). The main limitation of these instruments is their low resolution power and consequently the presence of spectral interferences caused by ions having the same nominal mass as the isotope measured (Marchante-Gayón et al., 1999). The interferences are particularly frequent and severe in the region under 80 Da, which includes most of the elements of biomedical interest.

Table 4. Q-ICP-MS

Advantages	Drawbacks
- multielement capacity - low detection limits - high sensitivity - wide linear range - isotope ratio determinations	- high cost - requires skilled personnel - suffers from spectral interferences

1.2.4 High Resolution ICP-MS

As mentioned above the Q-ICP-MS suffers from several types of non-spectral and spectral interferences, which constitute the principal limitations of the technique. Interferences in the analysis of complex matrices, such as human body fluids containing high amounts of inorganic and organic components are especially challenging. Many of these spectral interferences can now be overcome by high resolution ICP-MS, also called sector field or double focusing ICP-MS. The HR-ICP-MS combines a magnetic sector (mass focusing) with an electrostatic sector (energy focusing) which makes it possible to separate ions with the same mass-to-charge ratio but slightly different energies (Marchante-Gayón et al., 1999). The mass resolution offered by the HR-ICP-MS is sufficient to separate most of the common spectral interferences. The HR-ICP-MS is characterized by a low instrumental background, which yield superior detection limits at low resolution (Moens et al., 1995).

Table 5. HR-ICP-MS

Advantages	Drawbacks
- multielement capacity - very low detection limits - high sensitivity - high resolution - excellent linear range - low instrumental background - isotope ratio determinations	- high investment cost - high running and maintenance cost - requires highly skilled personnel

1.2.5 Trace element speciation

To elucidate the metabolism, bioavailability and toxicity of a metal, it may not be sufficient to determine the total concentration of the metal (de la Calle Guntiñas et al., 2002). Knowledge of the binding of trace elements to the different proteins, and other aspects of speciation of trace elements in biological systems is therefore of increasing interest. HR-ICP-MS is a very powerful detector for trace element speciation using e.g. high performance liquid chromatography (HPLC) or gas chromatography separations because of its selectivity and extremely high sensitivity (Bergomi et al., 2002; Wang et al., 1999; Gellein et al., 2007).

1.3 Challenges in sampling and analysis

1.3.1 Sample collection and contamination control

Contamination is by far the most important source of error in the determination of trace elements. Trace elements are ubiquitous in the earth's crust and may enter the samples at all phases of sample preparation and analysis. The increasing sensitivity of analytical methods has resulted in the use of decreasing amounts of the analyte in question, and the effect of even low-level contamination may be disastrous. Appropriate measures must therefore be taken to avoid contamination of the samples.

Sample collection is the first critical step in trace element analysis of human samples (Rodushkin and Ödman, 2001). Guidelines for sample collection in blood and urine have been developed (Cornelis et al., 1996), however such guidelines are not always possible to follow stricktly. When biological fluids such as blood and cerebrospinal fluid are collected, the use of needles is necessary. Stainless steel needles are commonly used in clinical practice. These needles can contaminate the sample, especially of Mn, Ni, Cr and Co (Aito and Järvisalo, 1994). It is therefore preferable to use a siliconized needle (Ericson et al., 1986). The collecting tubes for whole blood are equipped with self-sealing rubber stoppers that inevitably will come in contact with the blood. Such rubber products frequently contain metals as e.g. material modifiers. Anticoagulants are another source of potential contamination of blood samples. If possible, blood collection containers manufactured especially for trace element analysis should be used, and the needle should be flushed with at least 5-10 ml blood before the sample is collected.

Biological specimens undergo numerous transformations when kept at room temperature and preservation of samples before chemical analysis is therefore frequently necessary. A variety of preservation methods exist, such as low temperature (-20 or -70 °C), lyophilization, or the addition of chemicals (acids and preservatives). Formalin is an effective tissue preservative and is therefore often used (Meldrum, 2001). However, formalin can affect the sample by contaminating it as well as extracting elements from the sample (Gellein et al., 2008a). This should be considered when using formalin-fixed biological tissue in trace metal analysis. When possible, fresh/frozen tissue should always be considered as a preferable alternative.

Modern laboratories will inevitably be contaminated by dust from the ventilation ducts. Depending on the building design, the dust might originate from concrete and/or aluminium ducts. Dust from concrete will likely contain trace amounts of Cr and Ni while aluminium ducts may release particles containing Al and Zn. If samples are exposed to such dust it may introduce trace metal concentrations many times higher than expected within the sample.

In order to minimize blank signals it is crucial to ensure minimal exogenous contamination by use of purified acids, and resorting to clean-room facilities is also preferred. Ultrapure water must be used. All sample storage containers and other equipment should be acid washed polyethylene, polystyrene, polypropylene or Teflon – and storage must be under appropriate conditions.

Recognition of contamination has dramatically changed our view of the true concentrations of many trace elements in biological samples. It has been noted that published normal serum levels of e.g. Cr have fallen dramatically over the last 60 years (figure 2) – and the most probable explanation for this is the increasing awareness of sample contamination.

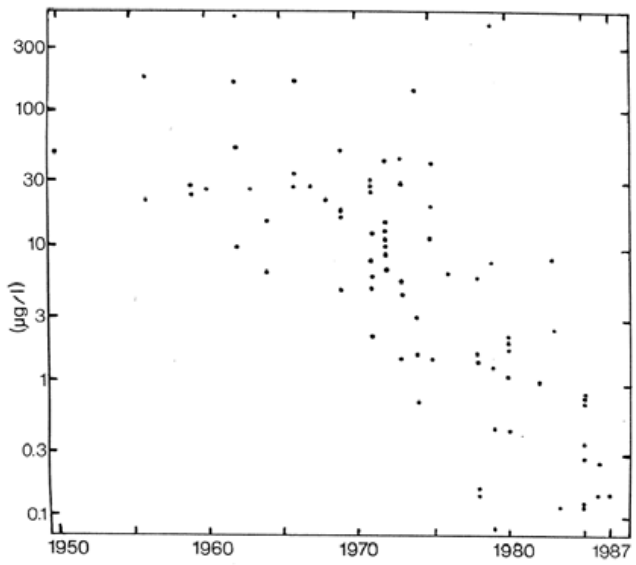

Figure 2. The apparent concentration of Cr in blood has decreased dramatically since 1950 (Flaten, 1988).

1.3.2 Sample preparation

Human biological materials have a range of both chemical and mechanical characteristics, e.g. hard, calcified tissues (e.g. bone, teeth), semi-hard tissue (e.g. hair, nails), soft body tissues and body fluids. In order to achieve a homogenous solution of such materials the digestion procedure must be designed with such differences in mind. Historically, acid digestion has been the most widely used technique for such preparation of biological samples. Oxidation is carried out by acids (HNO_3, $HClO_4$ and H_2SO_4 in various combinations and sometimes also with H_2O_2 or HF) combined with heating on a hot plate with temperatures up to 200 C (Vanhoe, 1993). Hot-plate dissolution procedures are limited by several factors: long dissolution times, the potential loss of volatile elements, sample contamination by excessive amounts of reagents, and prolonged contact with vessel materials (Kingston and Walter, 1998). A useful alternative to the hot plate is microwave heating. This method is now the dominant digestion method for human samples because it is more reproducible, more accurate, and less time consuming than conventional digestions on hot plates. Microwave systems keep blank levels low because only small volumes of reagents are required, and they allow more samples to be processed per hour than conventional digestion systems (Krachler et al., 1996). The digestion takes place in sealed quartz or Teflon bombs by heating in a microwave oven. The high internal pressure that develops ensures rapid digestion without any loss of analyte. Very little acid is lost during digestion so acid consumption can be limited and the blank kept minimal (Kingston and Walter, 1998).

The use of HNO_3 for digestion of biological samples is widely favoured, as it introduces less interference in the analysis of biological samples by ICP-MS than the other strong acids (Krachler et al., 1996). H, N and O do not add any additional spectral interference to those already existing in a pure water spectrum. This is in contrast to H_2SO_4 and $HClO_4$, which introduce polyatomic ions such as ClO and SO (Vanhoe, 1993). The oxidizing properties of nitric acid will dissolve most metals to free ions in solution. It has poor oxidizing strength at concentrations less than 2 M, but it is a powerful oxidizing agent in the concentrated form (14.5 M). Its oxidizing strength can be enhanced by the addition of e.g. hydrogen peroxide, or by increasing the reaction temperature and pressure (Kingston and Walter, 1998). Because hydrogen peroxide is reduced to H_2O, no additional interferences are introduced (Krachler et al., 1996). Complete digestion of tissue samples using microwave digestion requires at least 1 ml acid per 0.1 g tissue (wet weight). For whole blood, the ratio should be around 1:1 (v/v), but for serum or CSF the amount of acid can be halved. Because the concentrations of many trace elements in these materials are low, dilution should be kept to a minimum although the concentration of elements such as Na and K can be high enough to cause significant suppression of enhancement of the ion signal (Vanhoe, 1993).

After digestion, the samples must be diluted in order to get the appropriate acid concentration. Nitric acid concentrations in solutions should not be higher than 1.5 M for analyses by HR-ICP-MS as the signal is dependent upon the acid concentration. Higher concentrations cause rather rapid and severe corrosion of the sample and skimmer cones. The optimal nitric acid concentration is about 1 M. Solutions with this concentration provide signals that are independent of nitric acid concentration and are less corrosive to the cones (Krachler et al., 1996). Dilution can be done either by weight or in volumetric flasks. Samples should be kept in capped containers in the refrigerator until the analysis is carried out.

1.4 Quality control

1.4.1 Precision and accuracy

In discussing chemical analysis, it is necessary to define and distinguish between the terms accuracy and precision. Precision is an expression of the repeatability of a measurement (Wolf, 1987). Generally, the precision of a measurement is readily determined by simply repeating the measurement. The term accuracy refers the closeness of a result to its true or accepted value (Wolf, 1987). Inaccuracy reflects the extent of systematic error in the

measurement. The accuracy is best determined by using a certified reference material. Figure 3 illustrates the difference between accuracy and precision.

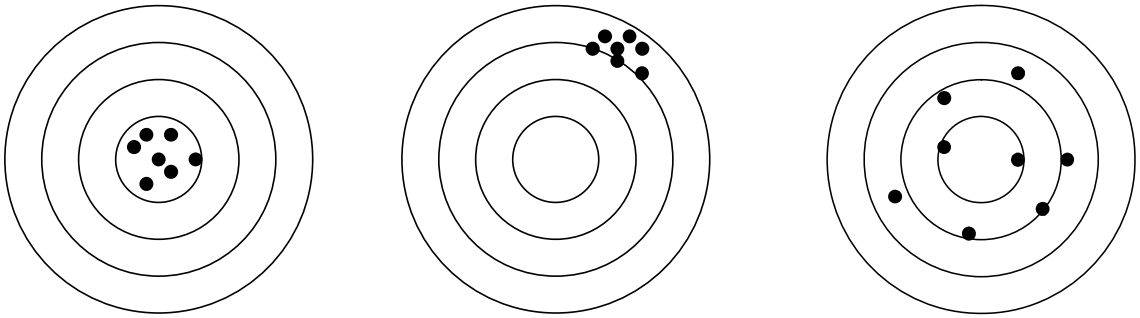

Figure 3. A: high accuracy, high precision, B: low accuracy, high precision, C: high accuracy, low precision

1.4.2 Reference materials

Reference materials are important for quality control of analytical measurements. They are used to determine the accuracy of the method. The ideal would be to have a certified reference material (CRM) for every type of matrix. This is however not always the case and one have to use another reference material with approximately the same matrix. For instance, a certified reference material for brain is unfortunately unavailable. Instead, bovine liver is often used as certified reference material in trace element analysis of brain tissue. Bovine liver contains 10% lipids and this is the closest to the (60%) lipid concentration in brain among the commercially available reference materials (Krachler et al., 1996). For whole blood, serum and urine there are CRMs available from several suppliers, while no CRM is available for cerebrospinal fluid.

1.4.3 Internal standard and matrix effects

Even though HR-ICP-MS instruments solve most problems caused by spectral interferences, they suffer from non-spectral matrix effects originating from the plasma and the sample introduction system. Internal calibration is one method to deal with this problem. Using internal calibration, the signal for one element is corrected or calibrated by using a second element as a reference point. An element with a known concentration, an internal standard, is then added to all solutions, including the blank, calibrators and unknowns. Ideally, this internal standard should

undergo the identical matrix suppression or enhancement as that of the analyte element. A close match of the mass number between the analyte and the internal standard is very important to effectively correct for matrix effects (Hsiung et al., 1997). The drawback of this method is that one can never be certain that the element that serves as an internal standard will undergo identical matrix effects as the element that is corrected. Addition of internal standard also increases the uncertainty of the measurement, as one more solution is added. An easier way to deal with this problem is to matrix match the standards used for calibration of the instrument. Sufficient dilution of the samples will also minimize the problem.

1.4.5 Memory effects

As a result of the high sensitivity and low instrumental background of the HR-ICP-MS, the instrument is highly susceptible to memory effects (Moens et al., 1995). Ways to deal with this problem is by carefully planning the sequence of the analysis. However, prolonged washing after e.g. standard solutions have been introduced to the instrument may also be necessary.

2 Summary of the individual papers

2.1 Paper I

The accurate determination of trace elements in body fluids and body tissue can be an important tool in the prevention and control of pollution as well as for the diagnosis and treatment of adverse health effects. The validity of the results obtained from trace element analysis however does not only depend on the analytical instrumentation, but also on the pre-analytical steps such as collection, preservation, storage, and treatment of the sample. There is frequently a need to preserve biological samples before chemical analysis. A common concern is that reagents, equipment or the environment may contaminate the samples, thus providing erroneous analysis. However, procedures may also cause loss of analyte from samples. This paper reports how trace element concentrations in biological tissue (brain samples) are effected by storage in formalin.

The concentrations of 20 trace elements were determined in formalin in which brain samples had been stored for different time durations ranging from weeks up to several years. The study shows that when biological tissue is stored in formalin, trace elements may leak from the tissue into the formalin solution. The leakage is time-dependent, and differs among the elements. For some elements such as Cr and Ni, the leaching is negligible, while for elements such as As, Cd, Mg, Rb and Sb, the leakage is considerable. For the essential elements Fe, Zn and Cu, the results are intermediate. It seems reasonable to assume that the differences depend on the strength and mode of binding of the different elements in the tissue. The potential for leaching should always be critically considered when using formalin-fixed biological tissue in trace element analysis. If available, fresh or frozen tissue should be used.

2.2 Paper II

The role and effects of trace elements in the human body strongly depend on the chemical form in which the element is present and e.g. binding to various proteins. To elucidate the metabolism, bioavailability and toxicity of a trace element, information about trace element speciation is needed. The objective of this paper was to develop procedures by which binding of trace elements to different proteins in cerebrospinal fluid (CSF) could be studied. The CSF surrounds the central nervous system and is in continuous equilibrium with the brain and the spinal cord. Trace elements and the metal-binding protein metallothionein (MT) have been implicated in the

pathogenesis of various neurodegenerative disorders. A method to study metallothionein and other metal-binding proteins would therefore be very useful.

Samples of CSF were collected from eight normal and healthy subjects. Proteins in the CSF samples were separated by size exclusion chromatography combined with high performance liquid chromatography (SEC-HPLC). The column was calibrated to separate proteins in the molecular weight range 6-70 kDa. The resulting fractions were then analyzed off-line using high resolution inductively coupled mass spectrometry (HR-ICP-MS) to determine the concentrations of the trace elements in the fractions.

The major challenge in this work was the very low concentrations, as only 100 µl CSF was injected to the column resulting in 35 fractions of 0.75 ml. It was possible to determine more than 10 elements of clinical interest in the CSF fractions. The study also showed that it is possible to detect MT based on the peaks where elements Cd and Zn are eluted because of the strong affinity of metals to MT. The method thus provides an opportunity to study MT and other metal binding proteins in CSF.

2.3 Paper III

Trace element analysis of human hair can be used for the assessment of environmental exposure and evaluation of nutritional status. Because trace elements are incorporated into the hair strand during growth, the analysis of hair strands provides an opportunity to monitor elements over a period from a few weeks to several years – depending on hair length. In this paper, the potential of human hair to indicate exposure or nutritional status over time has been evaluated by analysing trace element profiles in single strands of human hair. Hair strands from five healthy and occupationally unexposed subjects were cut into 1 cm long segments starting from the scalp. The hair segments were analysed by HR-ICP-MS. Profiles of 12 elements in single strands of human hair were obtained: Ag, As, Au, Cd, Cu, Hg, Fe, Pb, Se, Sr, U and Zn.

Hair is exposed to exogenous contaminants such as atmospheric pollutants, water, sweat and cosmetics. A challenge using trace element analysis of hair is therefore to differentiate between trace elements of internal and external origin. It was possible to correlate hair values and trace element intake for several elements, and valuable information about intake and exposure was obtained, especially for Hg, Se and Sr. For these specific elements, the

contamination risk is probably low, so that peaks and trends are easily detected independent of any pre-treatment procedures. These results substantiate the potential of human hair as a biomarker, even for subjects without any occupational exposure.

2.4 Paper IV

Imbalances in trace element status and exposure to toxic elements can cause adverse health effects. Trace element analysis of biological fluids gives us the opportunity to monitor and examine the trace element status of both healthy persons and patients. The objective of this study was to investigate whether changes in trace element concentrations could play a role in a rare familial neurodegenerative disorder called Skogholt's disease. Using HR-ICP-MS, 31 elements were determined in cerebrospinal fluid (CSF), blood plasma and whole blood from these patients, multiple sclerosis patients and a control group.

More than a threefold increased level of Cu and Fe, and a twofold increase in Zn were found in the CSF of Skogholt patients compared to controls. One of the characteristics of the disease is a highly increased protein level in CSF, and it is very likely that the elevated levels of these elements are due to a leakage of metal-binding proteins from the blood to CSF. Several other significant differences in trace element levels were also found, especially in the CSF.

The results from a study of trace elements in biological fluids can only establish whether there are any imbalances in trace element status of a patient group or an individual at that particular time. It is not possible to draw conclusions about the impact of these trace element imbalances on disease development and prognosis. This study shows how trace element analysis of biological fluids can be used to gain more information about a disease and possibly disease development.

2.5 Paper V

The aetiology of Parkinson's disease (PD) is unknown. Trace elements have been implicated in the pathogenesis of the disease and changes have been found for several elements in biological fluids from PD patients. Such changes could be a cause or a consequence of the disease. The objective of this study was to address this issue by analysing serum from PD patients collected prior to as well as after they were diagnosed with the disease.

Samples from 34 PD patients and 102 controls were received from the Nord-Trøndelag Health Study (HUNT 2). These samples were collected in 1995-97, 4-12 years before the PD patients were diagnosed with this disease. New samples from 19 of these patients were collected in 2007. Using HR-ICP-MS, 21 elements were determined in the serum samples. When trace element concentrations from preclinical PD patients were compared with controls, the only significant difference found was a slightly lower content of Hg in the patient group than in the controls. However, when trace element serum levels in patients from before and after they were diagnosed were compared, significantly higher levels were found for Hg, Ni, and Y, while Ca, Fe, Mg, Mn, Rb, and Se showed lower levels.

The results indicate that the illness may introduce changes in trace element homeostasis rather than the trace elements being a causative factor of PD. Such changes can be the result of biochemical characteristics of the illness itself or changes in environmental conditions associated with the illness. Even though the findings in this study do not provide information on how these changes affect the patients, it shows how trace element analysis of human serum can provide important information about disease development and progress.

3 Conclusions

Trace elements have a considerable impact on human health and understanding the optimal concentration of trace elements in the human body is therefore of great importance. The principal aim of this work was to demonstrate the potential use of HR-ICP-MS in biomedicine. Only when appropriate instruments and methods are applied can analytical chemistry be of practical use to determine important factors in the medical science.

Biological material is often available for analysis only in small or even minute amounts. The low detection limits, high sensitivity and multielement capability offered by HR-ICP-MS makes it possible to get accurate results for a wide range of elements in such samples. This has been clearly demonstrated in the study of trace elements in human hair, where 1 cm segments of a single hair strand with an average weight of 0.05 mg or less were analyzed. It was possible to obtain results for 12 elements in these minute samples and valuable time-dependent information about intake and exposure was obtained for Hg, Se and Sr. HR-ICP-MS was also utilized to develop a method to study protein-bound metals in cerebrospinal fluid (CSF) by coupling the HR-ICP-MS with a chromatographic method (SEC-HPLC). The major challenge in this work was again the small samples with very low concentrations It was possible to determine more than 10 elements of clinical interest in the CSF fractions. The separation was performed off-line in this work, but the HR-ICP-MS also offers the possibility of on-line coupling which would further enhance the chromatographic resolution.

Biological fluids are difficult matrices to analyse because they contain large amounts of proteins, organic molecules and inorganic salts. HR-ICP-MS offers sufficient resolution to eliminate spectral interferences, which makes it possible to analyse almost any type of matrix. In a study of patients with a rare and relatively unexplored neurodegenerative disease, trace elements in whole blood, plasma and cerebrospinal fluid were determined. Results were collected from 31 elements in all three matrices and related to the disease. In another study, trace element analysis of serum of patients with Parkinson's disease collected before and after they were diagnosed, gave information relevant to the disease progress. The results from this study indicate that previous reported trace element changes may be a result of the disease rather than a cause of the disease. It should be emphasized that the findings must be supplemented with indicators of biochemical mechanisms in order to provide information about how trace element imbalances may affect the patients. However, trace element analysis of biological fluids can, in appropriate combination with other studies, provide important information about disease development and progress.

In conclusion, the HR-ICP-MS is a very powerful technique for the determination of trace elements in biological samples. For most elements, the analytical technique is no longer the limiting factor. As demonstrated in paper I, the challenge is rather to control all steps from sample collection to analysis. Reagent blanks and memory effects must be reduced in order to make fully use of the possibilities of the instrument. The advantages of HR-ICP-MS over other techniques make it possible to achieve reliable and accurate results in all matrices. Thus, HR-ICP-MS offers a considerable potential for further understanding the role of trace elements in biomedicine.

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Paper I

Paper I

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Paper II

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BRAIN RESEARCH

Research Report

Separation of proteins including metallothionein in cerebrospinal fluid by size exclusion HPLC and determination of trace elements by HR-ICP-MS

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ABSTRACT

A method to study the protein binding patterns of trace elements in human cerebrospinal fluid (CSF) is described. Proteins in CSF samples were separated by size exclusion chromatography combined with high performance liquid chromatography (SEC-HPLC). The column was calibrated to separate proteins in the molecular weight range 6–70 kDa. Fractions were then analyzed off-line for trace elements using high resolution inductively coupled plasma mass spectrometry (HR-ICP-MS). We were able to accurately determine more than 10 elements of clinical interest in the CSF fractions. Results are presented for Cd, Mn, Fe, Pb, Cu and Zn. The total concentrations of 16 trace elements in human plasma and CSF are also presented. The method was able to differentiate the relative contribution of metallothionein and other proteins towards metal binding in human CSF.

1. Introduction

The central nervous system (CNS) is surrounded by cerebrospinal fluid (CSF). Thus, CSF is in continuous equilibrium with the brain and spinal cord and therefore detailed analysis of CSF has the potential to reveal important details and malfunctions in many diseases affecting the CNS (Hühmer et al., 2006). CSF is largely produced by the choroid plexus and it is distributed throughout the subarachnoid space surrounding the brain and the spinal cord as well as the interstitial space encircling cells in CNS. The total volume of CSF in humans is approximately 140 ml, with a mean production rate of 0.3–0.4 ml/min (Sickmann et al., 2002). The CSF contains small molecules, salt ions, peptides, proteins and enzymes that play critical roles in many physiological processes. Changes in CSF composition may reflect pathological processes in the CNS, and CSF therefore offers a unique window to study CNS disorders (Yuan and Desiderio, 2005).

Trace elements play numerous roles in biology, notably as integral parts of enzymes or protein structures (Smith et al., 1997). Metalloproteins are involved in electron transport, oxygen storage, metal transport, chemical bond hydrolysis, redox processes, and synthesis of biological compounds. Several trace elements are essential to human health, including iron, zinc,

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Table 1 – Calibration of the size exclusion column

Protein	T_E	MW
Albumin	10	66000
Metallothionein (MT-1+MT-2)	15	6500
Trypsin Inhibitor	16	24000
Lysozyme	21 and 25	14000
Insulin	26	5800

T_E=elution time in minutes.
MW=Molecular weight (Da).

copper, chromium, cobalt and molybdenum (Nordberg and Cherian, 2005). The physiological functions of trace elements in the human body depend on their binding to various proteins (Richarz and Brätter, 2002). To elucidate the metabolism, bioavailability and toxicity of a metal, it is therefore not sufficient to determine the total concentration of the metal (de la Calle Guntiñas et al., 2002). Also knowledge of the binding of trace elements to the different proteins in CSF is required. We are not aware of any published studies of speciation of trace elements in CSF.

Metallothionein (MT) is a family of proteins with low molecular weight (6500 Da) and a unique capacity to bind 7 metal ions through 20 sulfhydryl groups (Nordberg, 1998). Four major forms of MT have been described: MT-1, MT-2, MT-3 and MT-4 (Nordberg et al., 1971). MT is an inducible protein, and is known to play important roles in the toxicokinetics and biochemistry of essential as well as non-essential elements such as Zn, Cu, Cd and Hg (Nordberg and Cherian, 2005). Abnormalities in MT have been reported in multiple neurological disorders but definitive links remain to be established (Aschner and West, 2005). Metals such as zinc and copper also have been implicated as etiological factors in neurodegeneration. Taken together, these observations suggest that MTs may have important roles in several human neurological diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) (Johansson et al., 2005; Hozumi et al., 2004; Roos et al., 2006).

We wanted to develop procedures by which binding of trace elements to different proteins in CSF could be studied. By employing size exclusion chromatography combined with high performance liquid chromatography (SEC-HPLC), proteins in CSF-samples were separated according to size. The resulting fractions were then analysed off-line using high resolution inductively coupled mass spectrometry (HR-ICP-MS) to determine the concentrations of the trace elements in the fractions. Our main interest was to establish the procedures in order to test their usefulness in later studies of abnormalities in neurological diseases.

2. Results

2.1. *Molecular weight calibration*

Table 1 shows the retention times for the standard proteins used for calibration of the SEC-column. Albumin is the largest protein used for calibration and thus shows the shortest retention time. The smaller proteins then follow according to decreasing size. Lysozyme is made up by two different domains, four α helices form one domain and a β sheet forms the other domain (Stryer, 1997). Since lysozyme elutes with a double peak (21 min and 25 min), it seems probable that these two domains have been separated during the SEC. Fig. 1 shows the chromatogram for the calibration of the MT peak. The double peak is probably due to a partial overlap between MT-1 and MT-2. Wang et al. (2001) reported that the metallothionein peak had a retention time that corresponded to an apparent molecular weight of 13 kDa, instead of the true molecular weight of 6.5 kDa. Their explanation for this was the "dumbbell" shape of metallothioneins that results in the occupation of a larger volume than globular proteins with similar molecular weight. This is also in accordance with findings on Sephadex gel chromatography where MT elutes around 10 kDa which is explained by its Stokes' radius (Nordberg et al., 1972). The elution order

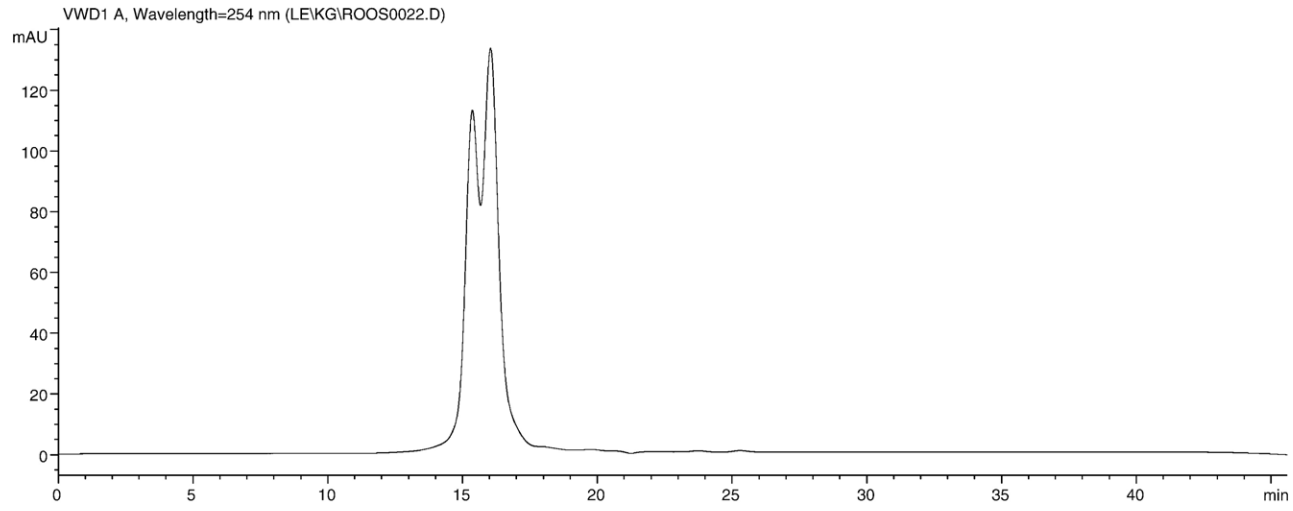

Fig. 1 – Elution profile of metallothionein by using HPLC with Superdex 75. Metallothionein from rabbit liver was applied to the column and fractions were collected each minute. Light absorbance (mAU) at 254 nm is on the ordinate. Time (minutes) is on the abscissa.

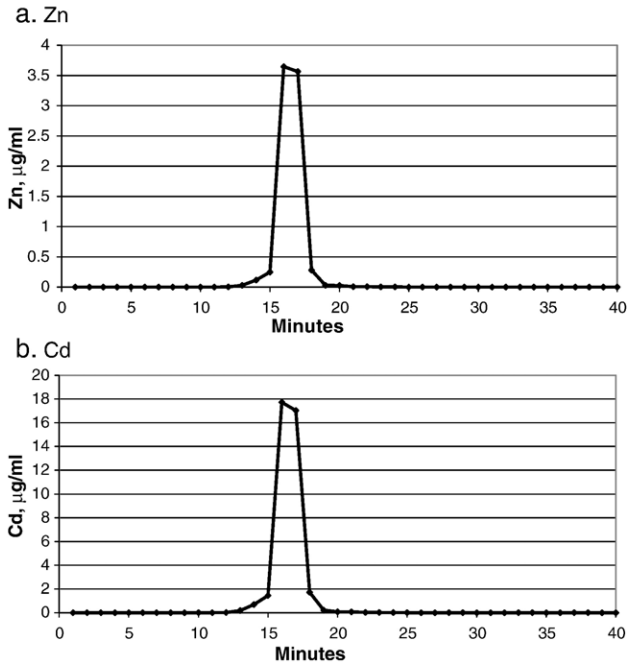

Fig. 2 – Zn and Cd concentrations as measured by HR-ICP-MS in fractions obtained by using HPLC with Superdex 75 and highly purified metallothionein. Note the high similarity of the patterns.

of SEC is based on volume of the protein and not strictly on the molecular weight. Thus metallothioneins often elute earlier than would be expected from their molecular weight (Wang et al., 2001).

2.2. *Trace element profile and reproducibility*

Figs. 2a and b show the profiles of Zn and Cd from the trace element analysis of metallothionein after fractionation by SEC-HPLC. The peaks are found at 15 min, and coincide with the retention time of MT. Zn and Cd both binds strongly to MT (Nordberg et al., 1971). The fact that both metals have the same elution time as MT shows that they still are bound to MT after passing through the column. In Figs. 3a–c the reproducibility of the method is illustrated. Two CSF samples from the same person were fractionated by SE-HPLC with a two-week interval and the fractions were analysed by HR-ICP-MS. Between the fractionations samples were stored in a refrigerator at 4 °C. Results are shown for Cd, Cu and Fe. The reproducibility is acceptable for the major peaks of these elements while there are some discrepancies for minor peaks indicating that sample storage is important. For Cu there is one main peak at 12–13 min, while both Cd and Fe elute mainly at 17–18 min. The peak width for these element selective chromatograms is similar to those obtained by measuring the UV-absorbance (λ=254 nm) (Fig. 4).

2.3. *CSF*

Fig. 4 shows a typical chromatogram of a CSF sample from a normal, healthy person recorded by UV-absorption at 254 nm. The first eluted peak in the chromatogram is albumin at 10 min. The majority of the proteins elute between 10 and 27 min. All CSF chromatograms also display a negative peak between 22 and 23 min. The size of this peak is equal in all chromatograms. The elution time of this peak corresponds to the elution volume of the column. At this volume the CSF fluid reaches the UV-detector. Since CSF has lower absorbance than the TRIS-buffer, a negative peak is obtained. We also observed the corresponding peak when water is injected into the SEC-HPLC.

Trace element concentrations in CSF from the same subject as Fig. 4 are presented in Table 3. The concentrations of Cd, Pb, Mn, Fe, Cu and Zn are shown for each fraction. The trace element profiles for different subjects show some variation, but generally the major part of all trace elements studied elute between 10 and 25 min. The total separation

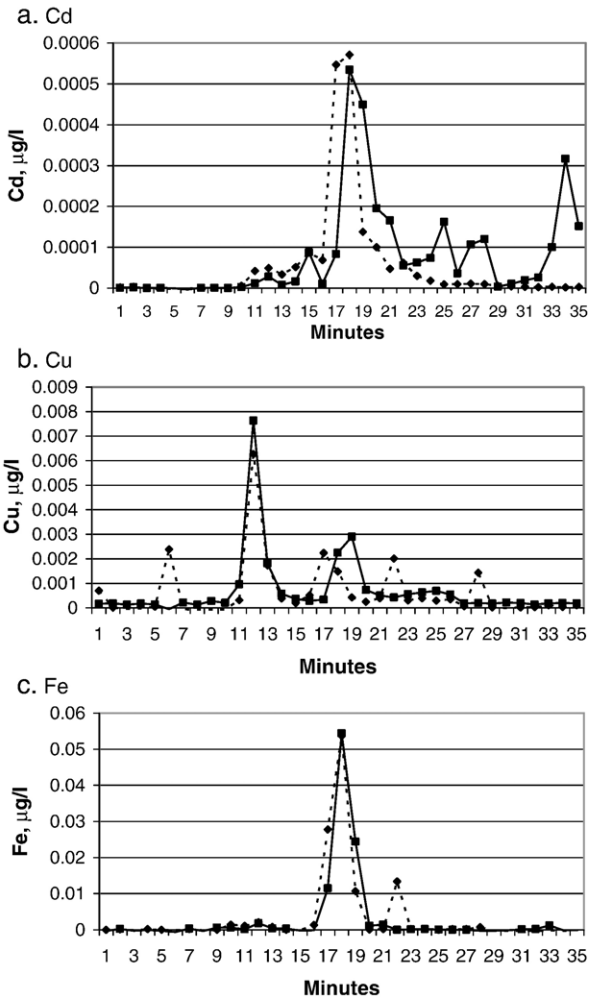

Fig. 3 – Reproducibility of the method shown by repeated separation of the same CSF sample fresh from freezer (solid line) and after 2 weeks stored at 4 °C (dotted line). 1 ml fractions collected and subsequent analysis by HR-ICP-MS. Elution time (in minutes) is given on the abscissa and concentration of the metals (µg/l) on the ordinate. µg/l=microgram per litre.

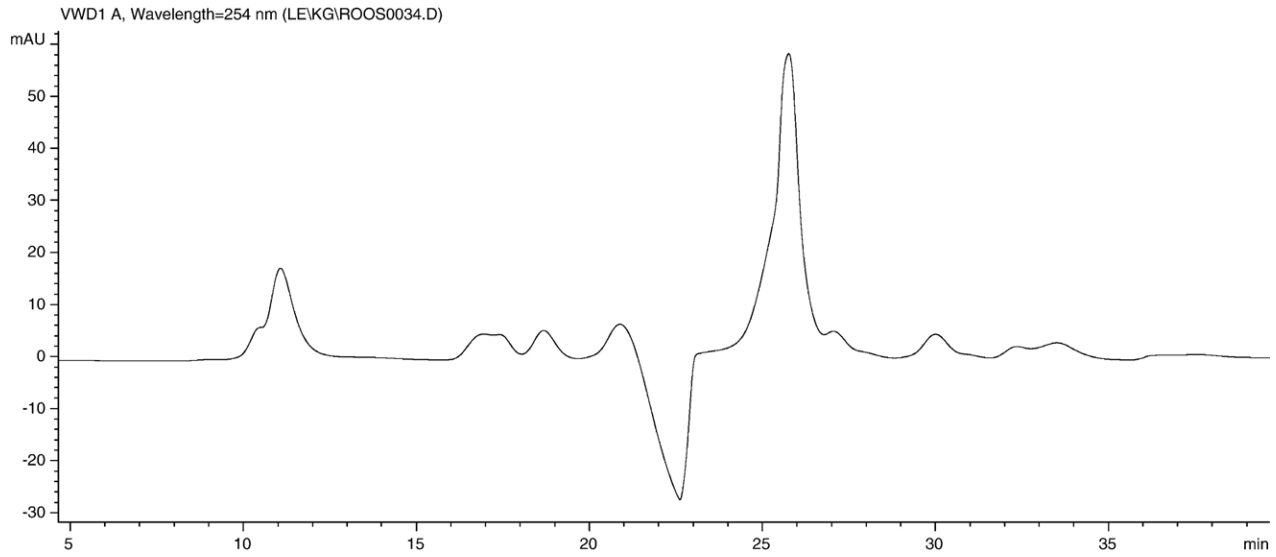

Fig. 4 – Chromatogram of CSF from a normal healthy subject using separation by SEC-HPLC. Light absorbance (mAU) at 254 nm is on the ordinate. Elution time (in minutes) is on the abscissa. Trace element composition presented in Table 3.

time for a sample is 40 min. Table 2 present concentrations of 16 trace elements in CSF and blood plasma for the study group.

3. Discussion

A reason for scarce reports of trace elements in CSF is that the concentrations of many elements are close to the detection limit. This paper describes new procedures to study trace elements in biological fluids, primarily metals in protein fractions separated by SEC-HPLC. The separation technique developed together with HR-ICP-MS analysis is very useful for studying metal containing proteins in body fluids also when metal concentrations are very low, which is the case especially after fractionation of CSF by HPLC, which inevitably entails a pronounced dilution (Prange and Schaumlöffel, 2002). HR-ICP-MS has technically indeed very low detection limits, so the operational detection limit is determined by the reagent blank. Thus, the technique is particularly useful for multielement analysis of small samples of biological material with low concentrations of trace elements. Furthermore, the eluents of the HPLC have to be tolerated by the plasma and the inlet system of the mass spectrometer, and high organic solvent concentrations or high salt concentrations cannot be used (Prange and Schaumlöffel, 2002). SEC-HPLC uses a non-denaturating mobile phase at physiological pH such as the TRIS-buffer, which stabilizes the original metalloprotein complexes and is easily tolerated by the HR-ICP-MS system (Prange and Schaumlöffel, 2002). No sample preconcentration is needed using this method.

A disadvantage of SEC-HPLC for MT analysis is that MT-1 and MT-2 isoforms cannot be distinguished (Lobinski et al., 1998). But better separations can be accomplished by using a longer column with a narrower molecular weight separation (Wang et al., 2001).

Desorption of weakly bound metals from proteins as well as adsorption to and release from a column may cause problems during HPLC separation of metalloproteins. However, no such problems were observed for the metals Zn and Cd bound to metallothionein, probably due to the strong coordination of Cd and Zn by mercapto groups (Hozumi et al., 2004). This can also be observed in Figs. 2a and b. This study shows that it is possible to detect MT based on the peaks where

Table 2 – Trace element concentrations in CSF and blood plasma from eight healthy subjects

Element	Unit	CSF		Serum	
		Mean	S.E.M.	Mean	S.E.M.
Cd	μg/l	0.09	0.02	0.14	0.03
Sn	μg/l	0.23	0.08	0.37	0.08
Hg	μg/l	0.32	0.12	0.46	0.11
Pb	μg/l	1.32	0.2	<d.l.	<d.l.
Mg	mg/l	29.2	0.8	21.9	0.8
Al	μg/l	27.68	4.29	13.7	1.42
V	μg/l	0.08	0.01	0.06	0.00
Cr	μg/l	0.44	0.09	0.29	0.06
Mn	μg/l	2.32	0.54	0.62	0.08
Fe	μg/l	74.8	25.2	1044	76
Ni	μg/l	5.64	2.14	0.63	0.26
Cu	μg/l	22.3	2.23	1129	124
Zn	μg/l	58.6	17.2	746	44.3
Rb	μg/l	47.5	3.2	166	13.1
K	mg/l	112	3	162.5	6
Se	μg/l	1.60	0.15	80.8	4.44

Average is the arithmetic average and S.E.M. is the standard error of the mean.
μg/l=microgram per litre.
mg/l=milligram per litre.

Table 3 – Trace element concentration in fractions of CSF after SEC-HPLC

Minutes	Cd	Pb	Mn	Fe	Cu	Zn
	100*μg/l	100*μg/l	10*μg/l	μg/l	10*μg/l	10*μg/l
1	3.3	1.8	0.0	0.2	0.1	1.6
2	3.4	1.2	0.0	0.0	0.0	0.0
3	3.6	2.3	5.0	0.0	6.9	0.9
4	2.9	1.0	12.9	0.0	0.0	0.0
5	2.0	0.2	22.9	0.2	0.0	0.4
6	0.0	0.0	1.0	0.0	1.2	0.0
7	0.0	0.0	0.0	0.0	0.5	0.1
8	0.0	0.0	0.0	0.0	0.0	0.2
9	0.0	0.0	0.0	0.0	0.0	0.0
10	0.0	0.0	0.0	0.4	0.0	0.6
11	0.6	0.0	0.3	0.1	0.4	0.0
12	0.8	0.0	0.7	1.0	8.1	0.0
13	0.2	0.0	0.3	0.5	2.5	0.0
14	4.1	0.0	1.1	1.1	1.1	0.0
15	9.8	0.0	1.0	0.0	0.6	0.0
16	11.6	0.0	1.3	0.0	1.4	3.2
17	15.2	0.0	0.4	62.6	0.4	0.0
18	14.5	7.9	20.1	97.9	6.6	4.0
19	12.4	8.8	26.9	18.9	0.2	0.0
20	5.2	0.0	0.0	4.8	0.1	176.2
21	3.0	0.0	0.0	1.3	0.0	0.0
22	2.4	0.0	0.0	0.8	0.0	0.0
23	1.7	0.0	0.0	0.2	0.0	0.0
24	0.8	0.0	0.0	0.3	0.6	0.0
25	0.6	0.0	0.0	0.2	0.0	0.0
26	0.3	0.0	0.0	0.6	0.0	0.0
27	0.1	0.0	0.0	0.7	0.0	0.0
28	0.0	0.0	0.0	0.1	0.0	0.0
29	0.0	0.0	0.0	0.0	0.0	0.0
30	0.0	0.0	0.0	0.0	0.0	0.0
31	0.0	0.0	0.0	0.4	0.0	0.0
32	0.0	0.0	0.0	0.0	0.0	0.0
33	0.0	0.0	0.0	1.2	0.0	0.0
34	0.0	0.0	0.0	0.0	0.0	0.0
35	0.0	0.0	0.0	0.0	0.0	0.0

Measured values below the detection limits (3*S.D. of the blank reading) are denoted as 0 in the table. Note that values for some elements have been adjusted by a factor (row 2) to enhance the readability of the table. The data are from one subject.
UV-absorption profile presented in Fig. 4.
*=multiplied.
μg/l=microgram per litre.

elements Cd and Zn are eluted as these metals have an extraordinary high affinity to MT.

4. Experimental procedures

4.1. Subjects

CSF was collected during 2005–2006 from outpatients enrolled from the waiting list for routine spinal tap at the Department of Neurology, Ullevaal University Hospital, Oslo, Norway. These were outpatients with minor or unspecific complaints such as headache, concern about having a neurological disorder, tingling sensations in a limb or suspected tick bite. A full medical history was recorded for each patient through the neurological follow-up. Among these patients CSF was accepted as control only if the final workup showed no signs of a neurological disorder. The spinal tap was thus performed on patients receiving spinal tap for other reasons than this study. Aliquots were taken aside for the present study. Informed consent was received from each patient in writing. Table 4 shows indication for spinal tap and final diagnosis for each patient. Occupation and smoking status as well as sex and year of birth is also shown.

Ethical approval for this study has been granted by the ethical committees in Oslo (470-03140) and Stockholm (03-353).

Table 4 – Clinical characteristics of subjects

	Sex	Born	Indication	Diagnosis	Smoker	Occupation
1	M	1947	Concern	None	No	Salesperson
2	F	1979	Headache	Neck pain	No	Preschool teacher
3	F	1928	Muscle twisting	Brain stem infarction	No	Nurse
4	F	1927	Arm pain	Arm pain	No	Housewife
5	M	1941	Paresthesias	Diabetes	No	Clerk
6	F	1975	Tick bite	None	Yes	Welfare officer
7	M	1974	SAH*	Amaurosis fugax	No	Civil engineer
8	F	1977	Concern	None	No	Train hostess

*Subarachnioidal hemorrhage.
M=male, F=female.
Smoker: current status of smoking.
Occupation equals occupation at the time of examination.

4.2. Sample collection

Cerebrospinal fluid (CSF) was collected using a standard Spinocan syringe 0.9×88 mm with Quincke cut provided by B.

Table 5 – Detection limits and determined concentrations of trace elements in the standard reference material Seronorm Serum Level 1

Element	Average values	Certified values	IDL	DL
Al	30.0	36.4	0.2	4.11
Cd	0.39	0.39	0.002	0.014
Co	0.23	0.23	0.004	0.002
Cr	0.7	0.7	0.005	0.104
Cu	987	1060	0.025	0.73
Fe	1247	1300	0.02	2.29
Hg	0.8	0.9	0.001	0.0007
K	175473	155000	40	11.0
Mg	20859	20000	0.35	4.84
Mn	8.7	8.3	0.006	0.176
Ni	5.7	5.9	0.013	0.74
Pb	0.7	0.9	0.002	1.719
Rb	6.1	7.1	0.012	0.045
Se	65.7	72.8	0.25	0.031
Sn	0.91	1.05	0.01	0.070
V	0.9	0.8	0.003	0.010
Zn	1086	845	0.025	12.4

All values are given in μg/l.
IDL=instrumental detection limit.
DL=detection limit given as 3*S.D. of blank samples.

Braun AG (Germany). Spinal tap was performed in a dedicated patient room with operation theatre cleaning routines. Tubes were thoroughly rinsed with ultrapure water (Elga, England) before use. Skin preparation included washing with 4% chlorhexidine gluconate in ethanol without phenol red. Liquor was collected dripping into six 1.8 ml polypropylene CryoTubes with silicone gasket (Nunc, Denmark), and immediately frozen.

Whole blood was collected into 7.5 ml Lithium-heparin polypropylene tubes dedicated for metal analysis (Sarstedt S Monovette system) using a 21G needle (Sarstedt, Germany). After centrifugation at 3000 rpm for 10 min, plasma aliquots of 1.5 ml were transferred into four CryoTubes using a clear plastic pipette. Tubes for CSF and plasma were thoroughly rinsed with ultrapure water (Elga) before use. CSF and plasma samples were stored at −20 °C or below awaiting further metal and protein analyses.

4.3. *Fractionation of CSF by SEC-HPLC*

A size exclusion column, Superdex 75 (10/300GL, Tricorn, Sweden), and HPLC system (Hewlett Packard, series 1050, USA) with quaternary pump, degasser, manual injector (100 μl loop) and a UV-detector (254 nm) was used for the separation of proteins in the CSF samples. Pump speed was set at 0.750 ml/min, and 0.02 M Tris-(hydroxylmethyl)-aminomethane (Tris, 1.08381.0500, Merck) with pH adjusted to 7.4 with 65% HNO_3 (1.00441.1000, Merck) was used as the mobile phase. Fractions (1 min per fraction) were collected in 5 ml sterile tubes (Falcon, cat. no. 352063) using a fraction collector (Gilson FC 203B). The CSF samples were injected directly and untreated into the HPLC equipment. Before the first sample each day and between each sample the column was washed with at least 2 volumes of the mobile phase.

The Superdex column was calibrated using proteins with known molecular weights ranging 6–66 kDa (Table 1); insulin from bovine pancreas (MW=5.8 kDa, I5500, Sigma), lysozyme from chicken egg white (MW=14 kDa, 62971, Fluka), trypsin inhibitor (MW=24 kDa, T9128, Sigma) and albumin (MW=66 kDa, A4503, Sigma). To calibrate the column for metallothionein, freeze dried liver MT (4.8 mg, MT-1+MT-2, MW=7 kDa) had been prepared by one of the authors from rabbit liver (Nordberg et al., 1972) and dissolved in Tris (0.02 M, 1 ml, pH 7.4) to a final concentration of 0.24 mg/ml. This concentration gave a narrow and defined double peak at 15 min elution time. The double peak is probably due to a partial overlap of MT-1 and MT-2. Fractions were subsequently analysed by "off-line" HR-ICP-MS for Cd, Cu, Fe, Mn and Zn. Reproducibility of the chromatography separations was checked by comparing repeated runs of the same sample.

4.4. *Sample preparation for trace element analysis*

Before trace element analysis samples from the HPLC fractionations were added 0.1 ml conc. HNO_3 (Scanpure, Scanlab) and diluted with 1.65 ml ultrapure water (Elga, England) using a calibrated pipette. Samples were then decanted into 14 ml tubes suitable for HR-ICP-MS analysis (Falcon, cat. no. 352059).

Plasma and CSF samples were also analysed for total trace element content. The samples (1 ml) were digested by adding concentrated HNO_3 (1.0 ml, Scanpure, Scanlab, Norway) directly in 14 ml polypropylene tubes (Falcon) and then digested on a block heater at 110 °C for 1 h. After digestion, samples were diluted with ultrapure water (Elga, England) to a final acid concentration of 0.6 M.

4.5. *Trace element analysis*

HR-ICP-MS analyses were performed using a Thermo Finnigan model Element 2 instrument (Germany). The radio frequency power was set at 1400 W. The samples were introduced using a CETAC ASX 500 autosampler with a peristaltic pump (1 ml/min). The instrument was equipped with a concentric Meinhart nebulizer connected to a Scott PFA spray chamber, platinum skimmer and interface cones and a quartz burner with a guard electrode. The nebulizer argon gas flow rate was adjusted to give a stable signal with maximum intensity for the nuclides ^{7}Li, ^{115}In and ^{238}U. Methane gas was used to minimise interferences from carbon and to provide enhanced sensitivity (Rodushkin et al., 2005).

The instrument was calibrated using 0.6 M HNO_3 solutions of matrix matched multielement standards. Calibration curves using 5 different concentrations were made using these standards. To check for instrumental drift, one of these multielement standards with known metal concentrations was analysed for every 10 samples. Certified reference material (SPS-SW1, SPS-SW-2, Spectrapure, Norway) were analysed at the beginning and end of each analytical sequence.

4.6. *Quality control*

For ultra-trace element analysis of clinical samples, controlling and minimizing all possible sources of contamination is mandatory (Rodushkin et al., 2004; Rodushkin and Ödman, 2001). To assess possible contamination during sample preparation, appropriate blank samples of ultrapure water with HNO_3 were prepared using the same procedure as for the samples. All blank levels obtained were negligible.

The accuracy of the method was verified by analysing the certified reference material Seronorm Serum Level 1 (Sero, Norway). Values are given in Table 5 together with detection limits for the HR-ICP-MS. No reference material for trace element content of CSF is commercially available.

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Per M. Roos performed the medical examinations and collected the samples. Kristin Gellein and Lars Evje performed all the analytical work. Tore Syversen supervised the laboratory work. Monica Nordberg purified and supplied metallothionein. Kristin Gellein prepared the first draft of the manuscript. All authors contributed to the manuscript.

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Paper III

Paper III

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Paper IV

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Trace elements in cerebrospinal fluid and blood from patients with a rare progressive central and peripheral demyelinating disease

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Abstract

A hereditary neurological disease in a family in Norway has been reported recently. The disease, which we refer to as Skogholt's disease, is a demyelinating disorder of both the central and the peripheral nervous system with adult onset. We investigated whether changes in trace element concentrations could play a role in Skogholt's disease. Using high resolution inductively coupled plasma mass spectrometry, we determined 31 elements in cerebrospinal fluid (CSF), blood plasma and whole blood from these patients, multiple sclerosis patients and a control group. More than threefold increased levels of Cu and Fe, and a twofold increase in Zn were found in the CSF of Skogholt patients compared to controls. Several other significant differences in trace element levels were also found. The increased levels of Cu and Fe in CSF may indicate an active role of these metals in the pathogenesis of Skogholt's disease. Apparently, these metal ions are transferred into the CSF through their protein chelation, as raised protein levels were also seen. We suggest that redistribution of metals from transport proteins into vulnerable sites in the central (and peripheral) nervous system may initiate critical lesions.

Keywords: Copper; Iron; Zinc; HR-ICP-MS; Multiple sclerosis; CSF proteins; Albumin; Skogholt's disease

1. Introduction

One of us (J.H.S.) recently discovered a family in Norway with a neurological disease, here referred to as Skogholt's disease, which apparently has not been found elsewhere. The disease has so far been diagnosed in 3 generations and has been thoroughly described by Hagen et al. [1]. It is a demyelinating disorder affecting both the central and the peripheral nervous system and it is different from previously described hereditary demyelinating disorders [1,2]. The symptom onset varies from before 30 to after 50 years of age, and the disease is uniformly gradually progressive. The symptoms are typically a gradually developing distal sensory loss, distal atrophy of extremity muscles or weakness of muscles in all extremities, unsteady gait, dysarthria, cognitive slowness, and memory impairment. Some patients have recurrent episodes of cerebral ischemia. The previously studied patients had increased cerebrospinal fluid protein levels [1]. Causes and mechanisms of this disease are unknown.

Considerable evidence implicates transition metals and other trace elements in the pathogenesis and/or aetiology of several neurodegenerative diseases [3,4]. Manganese has long been known as a neurotoxic trace element [5], as overexposure can cause manganism, a neurological syndrome that resembles Parkinson's disease [3]. Furthermore,

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considerable evidence suggests a role of the transition metals Cu and Fe in the pathogenesis of various neurodegenerative diseases, either by overexposure or deficit, and/or by disturbed inter- or intracellular metal trafficking [6]. Changes in the expression of metal-binding proteins have also been observed [7].

Skogholt's disease is an inherited disorder [1,2]. Several inherited neurological disorders in which metals are involved in the pathogenesis have been described [8–11]. In both Wilson's disease [8] and Menkes' disease [9], copper transport is disrupted, and demyelination is observed in the affected cerebral loci of patients with Wilson's disease [12].

The primary aim of the present study was to investigate whether changes in trace element concentrations could play a role in Skogholt's disease. Through common disease mechanisms, the results from this study may also be relevant for the pathogenesis of other demyelinating disorders such as multiple sclerosis (MS). Using HR-ICP-MS (high resolution inductively coupled plasma mass spectrometry) [13], we determined 31 elements in CSF, blood plasma and whole blood from these patients, MS patients and a control group. We found substantially raised Cu and Fe levels in CSF of Skogholt patients, which might suggest a role of these metals in the pathogenesis of the disease.

2. Materials and methods

2.1. Subjects

All study participants live in the county of Hedmark in the eastern inland part of Norway, their clinical characteristics are given in Table 1. Ten patients (6 men and 4 women) diagnosed with Skogholt's disease were included in the study. These were all the patients that were physically capable of travel and thus available for sampling of CSF and blood. Nine subjects diagnosed with multiple sclerosis were also included in the study. The control group comprised 13 individuals (5 males and 8 females) with no known neurological disease.

Table 1 lists the diagnoses, age and sex of all participants, together with information about daily consumption of coffee (tea was rarely consumed), smoking habits, physical activity and education collected from questionnaires filled out by the subjects during the diagnostic assessment. There is a good match in gender between the Skogholt and control group, but the MS group consists of 90% women. There were higher percentages of smokers both in the Skogholt group (60%) and in the MS group (78%) than in the control group (46%).

Written consent was obtained from each participant and the project has been approved by the Regional Committee for Medical Research Ethics in Norway (Region East, ref. no. 556-04224). Subjects were interviewed using a standardized form as to their life style, dietary habits and medical history. None of the participants in the study have had known occupational exposure to trace elements.

2.2. Sample collection and treatment

Samples were collected from patients in connection with diagnostic assessment at Sykehuset Innlandet Elverum, Department of Neurology. Maximum care was taken to avoid trace element contamination of the samples. The samples for trace element analysis were always collected after the samples for clinical–chemical analysis, to flush out the needles and tubing. The cerebrospinal fluid was obtained by lumbar puncture performed in the lateral recumbent position with

Table 1
Clinical characteristics of patients

Diagnosis	Age	Sex	Smoker	Coffee (L/day)	Activity	Education
Skogholt group						
Skogholt's disease	34	m	Y	0.3	Y	U
Skogholt's disease	40	m	Y	0.7	L	E
Skogholt's disease	32	m	Y	>1	N	H
Skogholt's disease	49	f	Y	1.0	L	E
Skogholt's disease	28	m	Y	>1	N	E
Skogholt's disease	44	f	N	0.3	N	H
Skogholt's disease	52	f	Y	1.0	Y	E
Skogholt's disease	55	f	N	0.4	Y	E
Skogholt's disease	57	m	N	0.4	L	E
Skogholt's disease	67	m	N	0.5	N	E
Mean age	45.8					
SEM (age)	3.9					
MS group						
Multiple sclerosis	60	f	Y	1.0	L	E
Multiple sclerosis	50	f	Y	1.0	Y	E
Multiple sclerosis	36	m	Y	>1	N	H
Multiple sclerosis	42	f	Y	0.4	Y	E
Multiple sclerosis	37	f	N	0	N	H
Multiple sclerosis	37	f	Y	0.5	Y	H
Multiple sclerosis	46	f	N	0.3	Y	H
Multiple sclerosis	37	f	Y	0.5	L	E
Multiple sclerosis	50	f	Y	1.0	Y	E
Mean age	43.9					
SEM (age)	2.8					
Controls						
Myalgia	48	f	N	0.6	N	E
Myelopathy	56	f	Y	0	Y	E
Myelopathy	50	f	Y	>1	Y	E
No findings	38	f	N	0	Y	C
No findings	36	m	N	0.5	L	E
No findings	34	m	Y	0.15	N	E
Paresthesia	57	m	N	0.2	N	H
Polyneuropathy	58	m	N	0.5	L	E
Cervical stenosis	60	f	Y	0.4	L	E
Bell's palsy	48	f	N	0.15	Y	U
Herpes zoster	78	f	N	0.1	Y	E
Cerebral infarcion	48	m	Y	1.0	N	E
Migraine	31	f	Y	0.5	L	E
Mean age	49.4					
SEM (age)	3.6					

f=female, m=male.
Smoker: Smoked regularly during the last 2 years.
Activity: physical activity classified as Y=yes, N=no, L=little.
Education: E=elementary school, H=high school, C=college, U=university.

a stainless steel needle (0.7×88 mm, Spinocan, B. Braun Melsungen). The CSF ran trough polyethylene tubing (Mediplast AB) into a polypropylene tube (8 mL, Sarstedt). The blood samples were obtained using a stainless steel needle (Becton Dickinson) and collected in tubes specially designed for trace element analysis (7 mL Vacutainer, sodium heparin, Becton Dickinson). All samples were stored at −20 °C until analysis.

2.3. Clinical–chemical analyses

All clinical–chemical analyses were performed at Sykehuset Innlandet Kongsvinger. Blood samples were analysed for haematological profile, transferrin, ceruloplasmin, F-T4, F-T3, TSH, cholesterol, triglycerides, HDL, protein, albumin and by electrophoresis for total protein, albumin and IgG. Protein analysis and electrophoresis were performed on CSF.

Prior to trace element analysis, all samples were digested by adding concentrated HNO_3 (Scanpure) directly in polypropylene tubes (Falcon, 14 mL) and then digesting on a block heater (110 °C, 1 h). For whole blood 1 mL HNO_3 was added to 1 mL of sample, for plasma 0.5 mL HNO_3 was added to 1 mL of sample and for CSF 0.5 mL HNO_3 was added to 0.5 mL of sample. After digestion, samples were diluted with ultrapure water (Elga) to achieve a final acid concentration of 0.6 M.

HR-ICP-MS analyses were performed using a Thermo Finnigan model Element 2 instrument (Bremen, Germany). The radio frequency power was set to 1250 W. The samples were introduced using a CETAC ASX 500 autosampler with a peristaltic pump (1 mL/min). The instrument was equipped with a concentric Meinhart nebulizer connected to a Scott PFA spray chamber, platinum skimmer and interface cones and a quartz burner with a guard electrode. The nebulizer argon gas flow rate was adjusted to give a stable signal with maximum intensity for the nuclides ^{7}Li, ^{115}In and ^{238}U. Methane gas was used in the analysis of CSF to minimise interferences from carbon and to provide enhanced sensitivity [14].

The instrument was calibrated using 0.6 M HNO_3 solutions of matrix matched multielement standards. A calibration curve using 5 different concentrations was made from these standards. To check for instrumental drift, one of these multielement standards with known metal concentrations was analysed for every 10 samples. Certified reference material was analysed at the beginning and end of each analytical sequence.

2.4. Quality control

For ultra-trace element analysis of clinical samples, controlling and minimising all possible sources of contamination is vital [15]. The potential contamination risk associated with devices routinely used in hospitals and clinical laboratories for sampling and storage of whole blood and serum is usually quite low [16]. To assess possible contamination during sample preparation, blank samples of ultrapure water were prepared using the same procedure as for the samples. All blank levels obtained were negligible.

The accuracy of the method was verified by repeated analyses of the certified reference materials Seronorm whole blood and Seronorm Serum Level 1 and Level 2 (Sero). The concentrations found were within 85–115% of the certified values. The precision of the method was checked by analysing

Table 2
Results from the clinical chemical analyses

			Skogholt (n=10)		MS (n=9)		Controls (n=13)	
		Unit	Mean	SEM	Mean	SEM	Mean	SEM
Serum	Albumin	g/L	43.5	0.9	45.0	0.7	44.1	0.6
	Protein	g/L	71.6	1.1	74.1	0.9	73.2	1.2
	Iron	µmol/L	15.2	2.3	18.9	3.2	18.8	1.8
	Transferrin	g/L	2.7	0.1	2.8	0.1	2.6	0.1
	Trf. Sat.	%	21.9	3.1	26.7	4.0	29.2	3.2
	Ferritin	µg/L	83.5	19.8	72.0	18.7	155	35.3
	F-T4	pmol/L	15.0	0.6	12.4	0.5	12.8	0.6
	F-T3	pmol/L	4.3	0.1	4.1	0.1	3.8	0.1
	TSH	mU/L	1.3	0.3	1.3	0.1	1.9	0.3
	Cholesterol	mmol/L	6.1	0.4	6.0	0.4	5.9	0.4
	TRiglyseride	mmol/L	3.0	0.6	1.2	0.2	2.0	0.3
	HDL-cholesterol	mmol/L	1.2	0.0	1.5	0.1	1.4	0.1
Serum electrophoresis	Total protein	g/L	72	1.1	74	0.9	73	1.2
	Albumin	g/L	44	0.9	45	0.7	44	0.6
	IgG	g/L	9.7	0.6	10.7	0.5	10.9	0.8
CSF electrophoresis	Total protein	mg/L	1218	112	319	20	360	45
	Albumin	mg/L	694	66	184	14	225	31
	IgG	mg/L	117	27	50	8	33	6

Mean denotes the arithmetic mean and SEM is the standard error of the mean.

Table 3
Trace element concentrations in plasma, whole blood and CSF of patients with Skogholt's disease, multiple sclerosis (MS) and controls

		Plasma						Whole blood						CSF					
		Skogholt		MS		Controls		Skogholt		MS		Controls		Skogholt		MS		Controls	
	Unit	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Ag	µg/L	0.07	0.02	*0.21*	0.05	0.06	0.01	0.09	0.03	0.24	0.06	0.12	0.04	<d.l.	–	<d.l.	–	<d.l.	–
Au	µg/L	0.021	0.004	0.006	0.003	0.009	0.008	0.039	0.006	0.022	0.003	0.016	0.003	<d.l.	–	0.037	0.014	0.023	0.006
Bi	µg/L	0.012	0.008	0.032	0.008	0.060	0.045	0.025	0.005	0.045	0.022	0.019	0.002	0.021	0.008	0.034	0.011	0.028	0.009
Ca	mg/L	39.1	0.8	39.9	0.8	39.9	0.4	51.1	1.7	49.6	0.9	51.4	1.3	59.6	5.5	58.0	1.2	57.1	0.9
Cd	µg/L	0.16	0.02	0.17	0.03	0.14	0.02	1.19	0.22	1.61	0.35	0.79	0.43	*0.013*	0.004	0.040	0.010	0.040	0.007
Ce	µg/L	1.44	0.06	1.50	0.04	1.46	0.06	13.74	1.07	11.63	1.81	11.02	1.50	<d.l.	–	0.002	0.002	0.002	0.002
Co	µg/L	0.072	0.009	0.075	0.014	0.066	0.007	*0.275*	0.017	0.273	0.030	0.214	0.017	<d.l.	–	<d.l.	–	<d.l.	–
Cr	µg/L	0.59	0.15	0.41	0.12	0.47	0.20	1.03	0.17	0.76	0.15	0.46	0.27	0.12	0.04	0.37	0.08	0.65	0.45
Cs	µg/L	0.37	0.05	0.36	0.04	0.33	0.03	3.78	0.43	3.31	0.43	3.40	0.22	0.29	0.04	0.33	0.02	0.30	0.02
Cu	µg/L	477	23	487	39	478	37	950	56	944	84	859	30	*69.3*	7.7	21.6	2.3	21.7	2.0
Fe	µg/L	440	57	453	73	458	36	460,000	14,800	474,000	11,400	454,000	15,100	*106.2*	11.8	19.8	2.6	30.5	9.7
Hg	µg/L	0.68	0.10	0.70	0.09	0.50	0.04	2.49	0.31	2.29	0.44	1.87	0.28	*0.79*	0.04	1.31	0.18	1.14	0.09
Li	µg/L	0.44	0.02	0.47	0.03	0.51	0.03	0.94	0.06	0.89	0.07	0.88	0.09	23.0	10.3	24.8	12.6	24.1	14.9
Mg	mg/L	8.59	0.19	9.05	0.23	9.02	0.17	35.3	1.0	35.1	1.1	34.7	1.1	37.3	1.5	36.7	0.5	36.5	0.5
Mn	µg/L	2.66	0.20	2.64	0.58	3.09	0.35	*8.94*	0.52	*9.34*	0.57	7.04	0.60	*0.79*	0.17	1.19	0.23	1.32	0.14
Mo	µg/L	0.61	0.03	0.57	0.03	0.78	0.11	*0.36*	0.05	*0.60*	0.13	1.07	0.20	0.25	0.02	0.22	0.04	0.42	0.10
P	mg/L	53.3	2.3	56.4	1.7	58.0	2.1	*458*	63	451	453	411	31.1	*22.4*	1.0	19.0	0.6	19.1	0.6
Pb	µg/L	0.51	0.09	0.53	0.13	0.93	0.21	22.3	14.0	18.6	11.5	20.3	8.5	0.28	0.09	0.76	0.13	0.59	0.07
Rb	µg/L	*72.7*	5.25	66.6	5.15	56.6	2.61	*2061*	133	1815	157	1560	75	86.9	6.4	76.7	5.2	65.4	3.8
S	mg/L	474	7.7	509	13.4	497	10.7	*1626*	56	1490	52	1515	29	*44.8*	4.2	13.8	0.7	16.0	1.6
Se	µg/L	42.9	2.3	45.4	2.1	41.1	2.2	152.8	7.8	157.6	13.7	145.0	6.5	*8.75*	1.21	2.28	0.30	2.48	0.21
Si	µg/L	51.4	20.3	54.0	15.6	62.7	12.2	1109	127	668	51	655	109	362	16	305	20	327	31
Sr	µg/L	11.1	0.7	11.2	1.0	11.7	1.0	16.2	1.1	15.2	1.0	16.3	1.4	15.4	1.3	15.1	1.1	15.9	1.4
Th	µg/L	0.015	0.003	0.013	0.003	0.014	0.002	*0.028*	0.004	0.020	0.003	0.015	0.002	0.008	0.001	0.025	0.008	0.021	0.004
Ti	µg/L	0.42	0.09	0.47	0.06	0.39	0.05	1.15	0.28	1.49	0.37	0.45	0.11	0.32	0.07	0.39	0.11	0.59	0.08
Tl	µg/L	0.008	0.001	0.008	0.001	0.007	0.001	0.037	0.002	0.033	0.003	0.030	0.003	*0.046*	0.002	0.086	0.011	0.077	0.005
U	µg/L	0.026	0.004	0.022	0.002	0.028	0.003	0.039	0.006	0.027	0.003	0.029	0.004	<d.l.	–	<d.l.	–	<d.l.	–
V	µg/L	0.056	0.005	0.064	0.004	0.066	0.006	0.032	0.008	0.054	0.008	0.048	0.012	0.021	0.008	0.034	0.005	0.059	0.004
W	µg/L	0.037	0.004	0.046	0.004	0.040	0.001	0.071	0.023	0.073	0.012	0.079	0.037	<d.l.	–	<d.l.	–	<d.l.	–
Y	µg/L	0.025	0.002	0.025	0.001	0.028	0.001	0.483	0.018	0.585	0.034	0.503	0.036	0.004	0.001	0.005	0.001	0.005	0.001
Zn	µg/L	333	17	333	16	342	15	5917	291	5897	286	5642	249	*42.0*	4.4	21.5	2.0	24.6	2.5

SEM=standard error of the mean; µg/L=micrograms/litre; mg/L=milligrams/litre; <d.l.=below the detection limit (defined as 3*SD of blank samples).
Values significantly different from controls are indicated in italics.

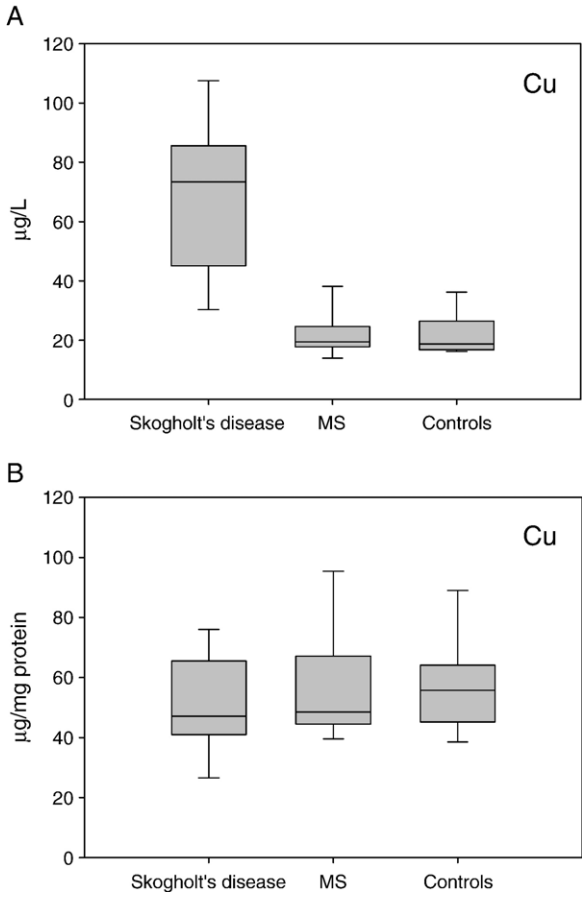

Fig. 1. Cu concentrations in CSF (µg/L). Boxes represent 75th (upper) and 25th (lower) percentiles, while the horizontal line indicates the median value. Vertical bars show the upper and lower extremes of the range (95th and 5th percentiles). A shows concentrations in µg/L, B in µg/mg protein.

four separate preparations of the same samples and it was better than 15% relative standard deviation for all elements.

In conclusion, the precision and accuracy of the HR-ICP-MS method was acceptable for all three types of body fluid. The concentrations of trace elements found in this study are among the lowest reported in human CSF [17–21], indicating that contamination of the samples has not been a major problem. Also for whole blood and plasma our values are in the lower range of values reported earlier [17–19,22–25].

2.5. Statistical evaluation

SPSS was used for statistical evaluation. Differences between the groups were tested using one-way analysis of variance (ANOVA) with post hoc multiple comparisons. At $p<0.05$ differences were considered significant.

3. Results

Table 2 shows that the level of serum ferritin is markedly decreased (about 50%) in both Skogholt and MS patients. All patients with Skogholt's disease have highly increased level of protein in the CSF, with values ranging between 0.80 and 2.24 g/L, which is almost 4 times higher than in the MS patients and controls. Protein levels this high are quite rare and may in fact be valuable as a diagnostic tool for this disease.

Table 3 shows the concentrations of the 31 elements in blood plasma, whole blood and CSF. Selected results for CSF are given in Figs. 1–6, both in µg/L and in µg/mg protein.

3.1. Blood

In whole blood, an increased level of Mn was found in MS patients as well as in Skogholt patients. Significantly increased levels of Co (30%), P (10%), Rb (30%), S (7%) and Th (85%) were also observed for Skogholt patients compared to controls. The level of Ag was higher for MS patients in both blood plasma and whole blood, although the difference was not significant in whole blood. A slightly increased level of Rb was found in both blood plasma and whole blood in the Skogholt patients. The level of Mo was lower in both Skogholt patients and MS patients compared to the control group.

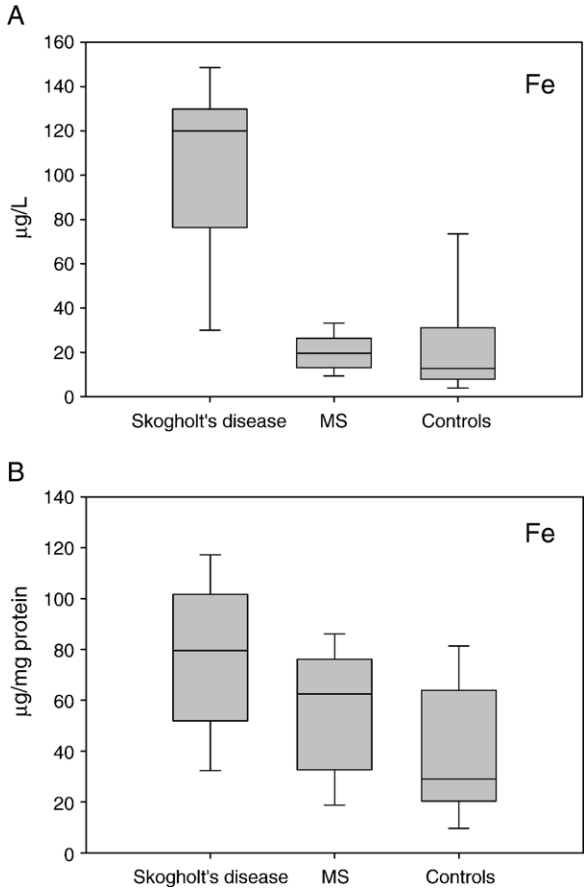

Fig. 2. Fe concentrations in CSF (µg/L). Boxes represent 75th (upper) and 25th (lower) percentiles, while the horizontal line indicates the median value. Vertical bars show the upper and lower extremes of the range (95th and 5th percentiles). A shows concentrations in µg/L, B in µg/mg protein.

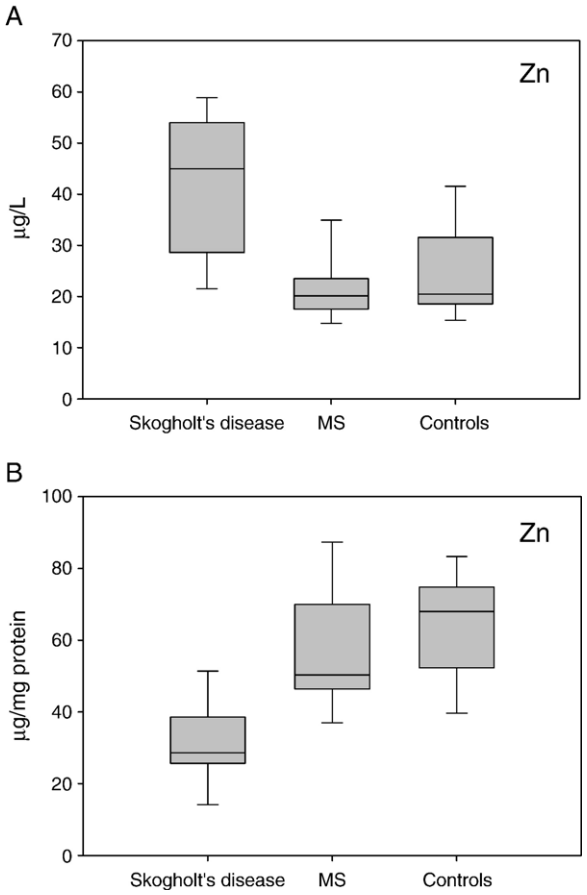

Fig. 3. Zn concentrations in CSF (µg/L). Boxes represent 75th (upper) and 25th (lower) percentiles, while the horizontal line indicates the median value. Vertical bars show the upper and lower extremes of the range (95th and 5th percentiles). A shows concentrations in µg/L, B in µg/mg protein.

3.2. CSF

The most striking finding in this study was a more than threefold increase in the concentrations of Cu and Fe, and a twofold increase in Zn in the CSF of Skogholt patients relative to controls. A parallel increase in Se and S was also observed. The levels of the toxic metals Cd, Hg and Tl were significantly lower in Skogholt patients compared to controls.

4. Discussion

The main finding in the present study is the highly increased levels of Cu, Fe and Zn in CSF from patients with Skogholt's disease. Whether these increased metal levels are a cause of effect of the disease is impossible to state at present, however as this finding is not reflected in blood it seems probable that the increased levels in CSF are not caused by increased environmental exposure.

One of the characteristic features of Skogholt's disease is a highly increased protein level in CSF, which was confirmed in this study. The increased protein level could be caused by leakage across the blood–brain barrier. Cu, Fe and Zn are known to be chelated to proteins. Thus, it seems likely that the increased trace element levels found in CSF occur as a leakage of protein chelates from blood to CSF. We were not able to find any apparent correlations between CSF concentrations of protein, Cu, Fe or Zn, and duration of the disease or the age of the patients. This suggests that a leakage of metal carrying proteins into the CSF may be an early pre-symptomatic event that could contribute to the disease process, rather than being a secondary consequence of the disease progression.

There were decreased levels of Hg and Cd compared to controls in CSF from Skogholt patients. These metals appear to be more tightly bound to high molecular weight selenoproteins than to albumin in the circulation and may thus avoid the diffusion into the CSF [26].

The only finding common to both the Skogholt and MS patients was a 30% increased level of Mn in whole blood. A limited number of studies have been published on the possible role of trace elements in MS [20,27–35]. Melø et al. [20] found increased Cu levels and decreased Mn in CSF in MS patients compared to controls, and no differences were

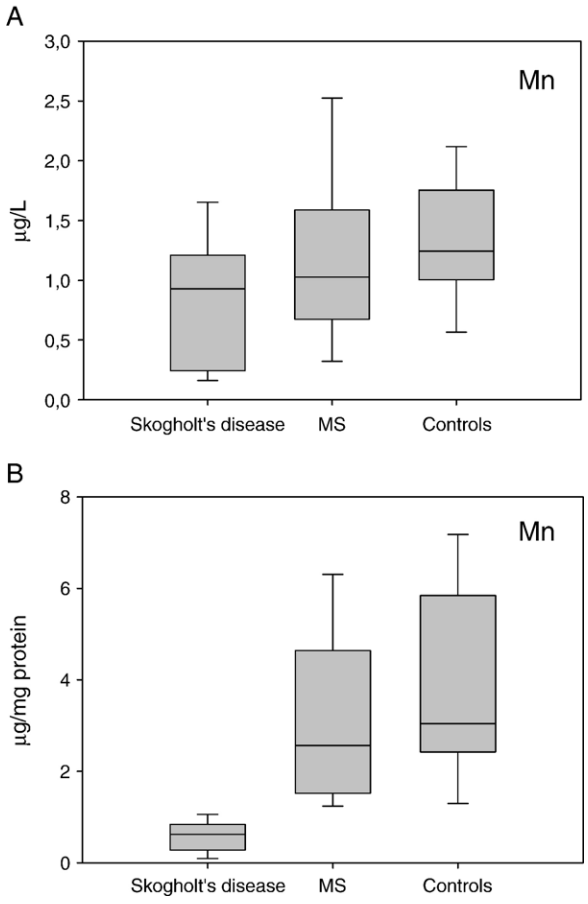

Fig. 4. Mn concentrations in CSF (µg/L). Boxes represent 75th (upper) and 25th (lower) percentiles, while the horizontal line indicates the median value. Vertical bars show the upper and lower extremes of the range (95th and 5th percentiles). A shows concentrations in µg/L, B in µg/mg protein.

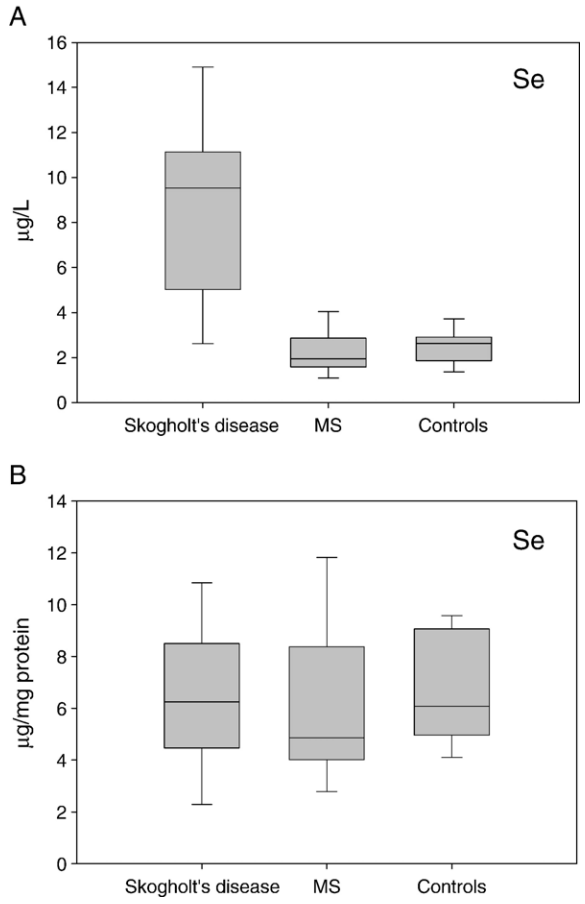

Fig. 5. Se concentrations in CSF (µg/L). Boxes represent 75th (upper) and 25th (lower) percentiles, while the horizontal line indicates the median value. Vertical bars show the upper and lower extremes of the range (95th and 5th percentiles). A shows concentrations in µg/L, B in µg/mg protein.

found for Zn. Normal values for Fe, K, Na, Rb, Ca and Mg have been reported in blood or CSF [30–34].

The substantial alterations in metal homeostasis observed in the Skogholt group might result in an increased level of free radicals [4,36,37]. A large body of evidence implicates crucial roles of oxidative stress in the pathogenesis of several neurodegenerative disorders [37,38], including multiple sclerosis [39]. The nervous system and in particular the myelin sheath seems to be more susceptible to oxidative damage than other organs, partly due to its high metabolic activities and rather marginal antioxidant defence systems and a relatively high content of vulnerable fatty constituents [36]. The progressive myelin destruction that characterizes Skogholt's disease and MS may be accelerated by oxidative stress.

Normally, most metals circulate in blood bound to proteins rather than as free ions [40]. Cu is bound to the specific Cu-transporter ceruloplasmin (90%) and to albumin (6%). In the same way Fe is bound in blood to transferrin (>99%), while zinc is primarily bound to albumin (65%) and to a high molecular weight α-macroglobulin (32%) [40]. Mn is bound to transmanganin and albumin [41]. CSF resembles an ultrafiltrate of blood plasma [42,43]. Small amounts of plasma proteins enter the CSF at the choroid plexus, but also through the circulation of CSF. Two major barriers, the blood–brain barrier and the blood-CSF barrier define the major entry and exit routes of components into the CSF from the circulation [43]. Similarly, a tight barrier also protects the peripheral nervous system. Thus, the physiological protein concentration in CSF is only about 0.5% of that in the blood plasma. However, the protein pattern is very different. Proteins are transferred from plasma to CSF by diffusion, and the barrier is more easily traversed by small proteins than by larger, so the fraction of smaller proteins are larger in CSF compared to blood [43].

The results from electrophoresis of CSF from Skogholt patients (Table 2) show that more than 50% of the total protein content is albumin, and about 10% is IgG. Since albumin is exclusively synthesized in the liver, an increased albumin concentration in CSF strongly indicates increased blood-CSF barrier permeability [43]. Albumin and IgG are proteins of rather low molecular weight, which penetrate the barrier more easily by diffusion than larger proteins. Both Cu and Zn levels were elevated in the CSF of Skogholt patients compared to controls (Table 3). Both metals have a high

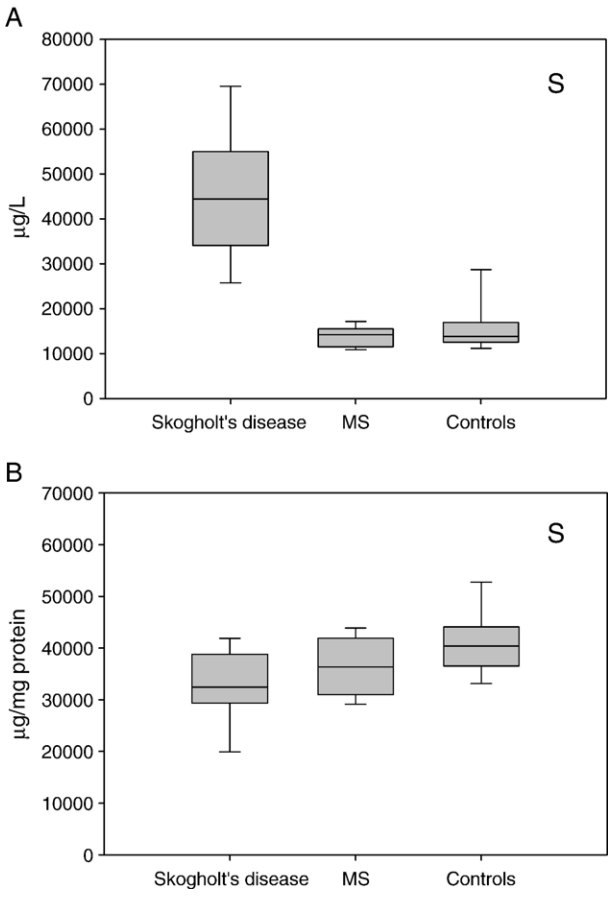

Fig. 6. S concentrations in CSF (µg/L). Boxes represent 75th (upper) and 25th (lower) percentiles, while the horizontal line indicates the median value. Vertical bars show the upper and lower extremes of the range (95th and 5th percentiles). A shows concentrations in µg/L, B in µg/mg protein.

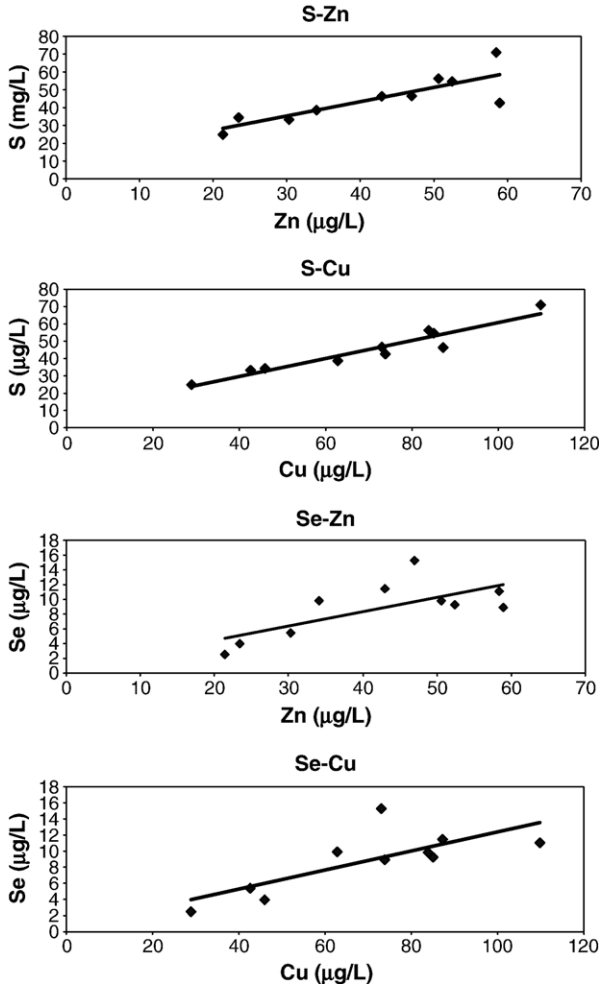

Fig. 7. Correlations between Cu and Zn, and S and Se, in the CSF of patients with Skogholt's disease.

affinity to albumin, which is almost comparably elevated in the CSF of the Skogholt group (Table 2). Therefore, it seems probable that Cu and Zn in CSF are transferred across the blood–brain barrier as albumin chelates. There were strong correlations between the protein level and the concentration of S and Se in CSF (Figs. 5 and 6). Cu and Zn are known to bind strongly to both selenium- and sulphur-containing ligands, which probably explain the strong correlations with S and Se for both Cu and Zn in the CSF (Fig. 7). While S and Se are incorporated into albumin as amino acids, Cu and Zn are reversibly chelated by the binding groups.

Metal ions carried by albumin or transferrin are readily exchangeable, as these proteins are the physiological transporters of metal ions to the tissues. When such metal-protein complexes are transported to target ligands with a higher affinity to the metals, a fraction of the metals may escape from their protein carriers and be redistributed to vulnerable sites. Vulnerable sites within the CNS may include myelin, and there are indications that metals may exert or aggravate toxic effects on myelin [44,45]. Toxic effects are of course more likely with long-term overexposure, as is the case for Cu, Fe and Zn in the CSF of the Skogholt patients.

All available evidence points to Skogholt's disease being hereditary [2]. Based on the results from the present study we hypothesize that a blood–brain barrier dysfunction is an early and presumably primary inherited defect, which causes the observed increases in metal concentrations. Whether the observed myelin degradation process might be causally related to the resulting long-term metal exposure deserves further exploration.

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Paper V

Trace elements in serum from patients with Parkinson's disease – a prospective case-control study.

The Nord-Trøndelag Health Study (HUNT)

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Running title:
Trace elements in Parkinson's disease

Abstract

To assess whether trace elements are involved in Parkinson's disease (PD) we have conducted a prospective study where 19 trace elements have been determined in serum collected from 33 patients before they were diagnosed with the disease, and 99 controls. As a follow-up, serum from 19 of the same patients collected 4-12 years after they were diagnosed with PD has been analysed. In the prospective part of the study, the only significant difference was a slightly lower content of Hg in the patient group than in the controls. In the follow-up, significantly higher levels of Hg, Ni, and Y, and lower levels of Ca, Fe, Mg, Mn, Rb and Se were found in the serum samples collected after the patients were diagnosed with PD compared with pre-diagnostic levels.

Section:

Disease-Related Neuroscience

Keywords:

Parkinson's disease; trace elements; epidemiology; prospective case-control study

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder that affects approximately 1% of adults older than 60 years. It is characterized by a progressive loss of dopaminergic neurons in the substantia nigra that is accompanied by a reduction in the synthesis of the neurotransmitter dopamine (Samii et al., 2004). The cause of the disease is still unknown but it seems likely that a number of aetiological factors are involved including ageing, genetic susceptibility, environmental exposure and lifestyle (Powers et al., 2003; de Lau and Breteler, 2006). In epidemiological studies, several factors have been reported to be associated with PD including age, exposure to toxicants (metals, pesticides and other neurotoxic substances), diet (sugar, vitamins, animal fats etc.), farming, non-smoking and family history of PD (Gorell et al., 2004). Cigarette smoking has consistently been found to be inversely associated with the risk of developing PD (Gorell et al., 1999). The risk of PD has also been reported to be inversely related to caffeine intake, both from coffee and non-coffee sources (Samii et al., 2004).

Transition metals such as Fe, Zn, Cu, and Mn are essential in many important biological reactions such as synthesis of DNA, RNA and proteins, and as cofactors of numerous enzymes. However, apart from Zn, these metals are also redox-active and have the ability to promote free radical formation that leads to oxidative stress. Considerable evidence implicates crucial roles of oxidative stress in the pathogenesis of major neurodegenerative disorders (Halliwell, 2006). The redox-active transition metals seem to play important roles in mediating the processes leading to oxidative stress, and thus may be involved in the neuropathology of disorders such as PD (Campbell et al., 2001).

A limited number of studies have addressed changes in serum trace elements in PD patients (Forte et al., 2004, 2005; Bocca et al., 2004; Hegde et al., 2004). To our knowledge, there are no data on trace element concentrations in body fluids of PD patients where the samples have been collected before they were diagnosed. Therefore, we conducted the present prospective study, utilizing serum samples collected in 1995-

1997 as part of the population-based second Nord-Trøndelag health study (HUNT 2) (Holmen et al., 2003), where about 75% of the population in Nord-Trøndelag county participated.

Changes in trace elements in PD patients could be a cause or a consequence of the disease. To address this issue, we obtained a second blood sample from 19 of the 33 PD patients included in the prospective study. Thus, these samples were collected 4-12 years after the patient was diagnosed with PD, and 10-12 years after the first samples were collected. Using HR-ICP-MS (high resolution inductively coupled plasma mass spectrometry) we have determined 19 elements in the serum samples.

2. Results

The characteristics of the PD patients are shown in Table 1. The table also shows for which patients a second blood sample was collected. Table 2 shows analytical detection limits and the results from analysis of certified reference sera.

Table 3 shows the trace element concentrations in serum from controls (n=99) and PD patients (n=33) before they were diagnosed with the disease. Results from the multiple conditional logistic regression analysis are given as odds ratios with 95% confidence intervals and p-values. In Table 4, the results from the follow-up are shown.

In the prospective part of the study (Table 3), the only significant difference was a slightly lower content of Hg in the patient group than in the controls. When trace element levels in patients from before and after they were diagnosed were compared (Table 4), significantly higher levels of Hg, Ni, and Y, and lower levels of Ca, Fe, Mn, Mg, Rb, and Se were found in the serum samples collected after the patients were diagnosed with PD. There were no apparent relations between trace element concentrations and duration of the disease (results not shown).

3. Discussion

The causes of PD are still largely unknown, but environmental factors seem to play a role in most cases, probably in interaction with susceptibility genes (de Lau and Breteler, 2006). Trace elements, and redox-active transition metals in particular, have been implicated in the pathogenesis of PD (Sayre et al., 1999). A large body of evidence implicates crucial roles of oxidative stress in the development of PD and other neurological diseases (Halliwell, 2006). Iron has more than other transition metals been implicated to undergo redox transitions leading to the generation of free radicals (Campbell et al., 2001). Increased levels of iron and oxidative stress have been found in different brain areas of PD patients, especially in the substantia nigra (Riederer et al., 1989; Gerlach et al., 1994). In this study, no difference in the level of serum Fe was found in the PD group before they were diagnosed compared to controls. However, the level of Fe was significantly decreased after diagnosis compared to prediagnostic levels. Decreased levels of Fe in serum of PD patients were reported in studies by Forte et al. (2005) and Hegde et al. (2004).

Copper has a functional role in many enzymes that require redox-reactions and an imbalance in the Cu homeostasis might lead to increased free radical production (Gaggelli et al., 2006). No significant differences were found for Cu (Tables 3 and 4). Forte et al. (2005) found a decrease in serum Cu in PD patients, while Hegde et al. (2004) found increased Cu levels in serum from patients with severe PD compared with controls.

Mn has long been considered as a possible contributor to PD because of its ability to cause manganism, a neurological disease with clinical signs similar to those displayed by subjects with PD (Calne et al., 1994). A significantly lower level of Mn was found in PD patients after diagnosis compared to prediagnostic levels. In two separate studies no difference in serum manganese was observed between PD patients and controls (Jiménez-Jiménez et al., 1995; Bocca et al., 2004).

The neurotoxic properties of Hg are well documented. Hg has the capability to damage neurons via a number of mechanisms (Aschner and Aschner, 1990). Bocca et al. (2004) reported a reduction of Hg in serum of PD patients compared to controls. In the present study a decreased level of Hg was associated with a decreased risk of Parkinsons disease according to the conditional logistic regression analysis (OR=0.69, p=0.03, Table 3). The level of Hg was however significantly higher in PD patients compared to prediagnostic levels (Table 4).

Ca and Mg are essential elements that are found in high amounts in serum. Little has been reported on the role of Ca and Mg in PD or related disorders, but a low intake of Ca and Mg has been implicated in parkinsonism-dementia on Guam (Garruto, 1991). Slightly but significantly lower levels of both elements were found in PD patients compared to prediagnostic levels. A lower level of Ca in serum of PD patients was also reported by Pino et al. (2005), while Forte et al. (2004) found a higher level of Ca. The same controversy also exists for Mg where Forte et al. (2004) found a lower level of Mg in serum from PD patients, while Hegde et al. (2004) reported a higher level.

Selenium is a well-known antioxidant, and there are some indications from animal models that Se may be protective in PD (Schweizer et al., 2004). However, we found no difference in serum Se levels between prediagnostic PD cases and controls (Table 3), but an average decrease of 34% in the PD cases between the two sampling times (Table 4). This may be at least partly related to the diseases process, although it should be noted that a slight decrease in serum Se with age has been reported in some studies (Versieck and Cornelis, 1989).

Increased levels were found in the PD patients compared to prediagnostic levels for Ni and Y, and a lower level was found for Rb (Table 4). We are not aware that any of these elements have been discussed as potential factors in the aetiology or pathogenesis of PD.

In summary, significant changes were found for several trace elements in serum collected from PD patients before they were diagnosed compared with levels in serum collected several years after they were diagnosed. However, when prediagnostic PD

patients were compared with controls no significant differences were found. Although it is possible that the levels of some trace elements in blood may change with age (Versieck and Cornelis, 1989), the observed differences may indicate that the illness could introduce changes in trace element homeostasis rather than the trace elements being a causative factor in PD. Such changes can be the result of biochemical characteristics of the illness itself or changes in environmental conditions (e.g. diet and physical activity) associated with the illness. There are no indications of significant changes in trace element exposure in the population of Nord-Trøndelag during the time between the first and the last collection of samples. However, as our study has relatively few patients, similar studies in other populations are warranted.

4. Experimental procedure

4.1. Subjects

All participants lived in the county of Nord-Trøndelag in the middle part of Norway at the time of sample collection in the HUNT 2 health survey in 1995-1997. Thirty-three patients (16 men and 17 women) diagnosed with idiopathic Parkinson's disease (PD) after 1995 were included in the study (Table 1). All PD patients were recruited among the outpatients attended in the neurology departments of two hospitals (Levanger Hospital and Namsos Hospital) and at one neurologist in private practice (Neurologist Duraj). All PD patients were assessed by neurologists and met the commonly accepted "UK Parkinson's Disease Society Brain Bank" diagnostic criteria for PD (Hughes et al., 1992). The control group comprised of 99 age (year of birth) and gender matched individuals (48 men and 51 women) with no known neurological disease. All subjects were thoroughly interviewed through the HUNT study in order to obtain detailed information about dietary habits, lifestyle and personal medical history, and we had access to all of this data. Written consent was obtained from each participant and the project has been approved by the Regional Committee for Medical Research Ethics.

4.2. Sample collection and treatment

A 7.5 ml blood sample was drawn from each participant. Serum was prepared by centrifuging at the screening site (3000 rpm, 10 min) and the centrifuged solution was immediately placed in a refrigerator. The samples were stored at -70 ºC until 2004 when aliquots of 300 µl were transferred to polypropylene tubes (14 ml, Falcon, USA) and frozen at -20 ºC until preparation for trace element analysis.

In 2007, we obtained a second blood sample from 19 of the 33 PD patients sampled in 1995-97. All samples were collected by the same procedure. Aliquots of 500 µl of serum were transferred to polypropylene tubes before samples were frozen at -20 ºC awaiting sample preparation.

Prior to trace element analysis, all samples were added concentrated HNO_3 (1:1 v/v) (Scanpure, Chem Scan, Norway) directly in the polypropylene tubes and digested on a block heater (110 ºC, 1 hour). After digestion, samples were diluted (1:24) with ultrapure water (Q-option, Elga, England) to achieve a final acid concentration of 0.6 M.

4.3. Trace element analysis

HR-ICP-MS analyses were performed using a Thermo Finnigan model Element 2 instrument (Bremen, Germany). The radio frequency power was set to 1400 W. The samples were introduced using a CETAC ASX 500 autosampler with a peristaltic pump (1 ml/min). The instrument was equipped with a concentric PFA-ST nebulizer connected to a Scott PFA spray chamber, platinum sample and skimmer cones and a demountable torch of quartz with a guard electrode. The instrument was calibrated by external calibration, using 0.6 M HNO_3 solutions of multielement standards, matrix matched by adding 100 mg/l Na. A calibration curve using five different concentrations was made from these standards. To correct for instrumental drift, one of these multielement standards with known metal concentrations was analysed for every 10 samples. All corrections were done manually after analysis. An aqueous certified reference material containing 66 elements (SPS-SW-2, Spectrapure Standards, Norway) was analysed as a quality control of the calibration of the instrument. To minimise

interferences, elements were determined in different resolutions; low resolution (m/ m = 400) (Hg, Pb, Tl, U, Y), medium resolution (5500) (Ca, Co, Cr, Cu, Fe, Mg, Mn, Mo, Ni, Rb, Sr, Zn) and high resolution (11,000) (As, Se).

4.4. Analytical quality control

The accuracy of the method was verified by repeated analysis of the certified reference materials Seronorm Level 1 and Level 2 (Sero, Norway). The concentrations found (Table 2) were within 90-115% of the certified values, except for Mo (81%), Y (83%) and Zn (124%) in Seronorm Level 1. The range of the analytical results (Table 2) is a measure of the precicion of the method. To assess possible contamination during sample preparation, blank samples of ultrapure water added HNO_3 (1:1) were prepared using the same procedure as for the samples. All blank levels obtained were negligible. Since no specific precautions to prevent trace element contamination were taken when the samples were collected in 1995-1997, we performed a leaching test of the cannulas and vacutainer tubes that were used. There were no indications of significant problems with contamination from either.

4.5. Data analysis

Using data from the first, prospective part of the study, we calculated the odds ratios (OR) for PD in relation to serum levels of trace elements using conditional logistic regression. In this analysis, we adjusted for the potential confounding effect of age, sex, coffee intake (cups per day), education (high school, college, university) and smoking habits (smoker, previous smoker, non-smoker). The precision of the estimated associations was indicated by 95% confidence intervals (CI).

In the second part of the study, a paired sample t-test was used to compare the levels of trace elements in serum samples from the same 19 patients collected prior to PD diagnosis with samples collected after diagnosis.

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Table 1. Characteristics of Parkinson's disease patients. Initial samples were collected during 1995-97. The second samples were collected in 2007.

	Born	Gender	Diagnosis	Second sample
1	1958	M	2001	+
2	1932	W	2000	+
3	1937	W	2001	+
4	1931	W	2001	+
5	1932	M	1998	
6	1936	M	1997	+
7	1930	W	1998	
8	1945	M	1997	+
9	1924	M	2002	+
10	1927	W	2000	+
11	1931	W	2001	
12	1945	W	1998	+
13	1938	W	2001	+
14	1928	W	2001	+
15	1937	M	2003	+
16	1952	M	1998	+
17	1952	M	2000	
18	1928	W	1996	
19	1928	M	1998	+
20	1919	M	2000	
21	1929	M	1998	
22	1936	W	1998	+
23	1928	M	1996	+
24	1937	W	1997	+
25	1941	W	2003	
26	1944	W	1996	
27	1932	M	1998	
28	1925	M	2003	
29	1945	W	1995	+
30	1929	M	1998	
31	1944	W	1998	
32	1936	W	1998	
33	1947	M	2003	+

M = man, W = woman

Table 2. Trace element concentrations in the certified reference serum Seronorm Serum Level 1 and Level 2 and detection limits for the HR-ICP-MS.

		Seronorm Level 1				**Seronorm Level 2**				**DL**
Element	Unit	Analysed		Certified		Analysed		Certified		Blank
	µg/l	Average	Range*	Average	Range*	Average	Range*	Average	Range*	
As	µg/l	0.341	0.28-0.40	<1.0		0.50	0.43-0.56	<1.0		0.0079
Ca	mg/l	104	98-111	105	98-112	127	117-135	117	106-126	0.1
Co	µg/l	0.25	0.23-0.26	0.23	0.21-0.25	3.0	2.8-3.2	3.20	2.9-3.4	0.0057
Cr	µg/l	0.84	0.73-0.94	0.7	0.60-0.80	4.9	4.7-5.0	5.2	4.0-6.4	0.0170
Cu	µg/l	1064	992-1136	1060	997-1123	2503	2400-2600	2600	2.4-3.4	0.268
Fe	µg/l	1335	1210-1459	1300	1200-1400	1888	1871-1903	1910	1790-2030	1.541
Hg	µg/l	0.90	0.9-1.0	0.90	0.8-1.0	2.00	1.92-2.08	1.86	1.61-2.11	0.0075
Mg	mg/l	22.0	20-23	20.0	18-22	31.4	29.3-33.4	28.90	27.3-30.5	1.115
Mn	µg/l	8.6	8.2-9.1	8.3	7.8-8.8	17.7	16.7-18.7	19.1	17.1-21.1	0.0090
Mo	µg/l	0.700	0.6-0.8	0.90	0.8-1.0	1.21	1.13-1.28	1.31		0.0002
Ni	µg/l	6.3	5.6-6.9	5.90	5.2-6.6	9.9	9.5-10.3	10.70	9.3-12.1	0.0231
Pb	µg/l	0.9	0.6-0.9	0.90	0.8-1.0	3.1	2.74-3.36	3.00		0.0231
Rb	µg/l	7.3	5.5-9.0	7.1	6.8-7.4	7.7	7.2-8.3	7.2		0.0027
Se	µg/l	67.0	63.3-70.8	72.8	66.7-79.8	154	146-162	136.0	127-145	0.0321
Sr	µg/l	94	86-101	102	97-107	131	125-137	130		0.0177
Tl	µg/l	0.046	0.040-0.051	0.0403	0.033-0.048	0.060	0.058-0.063	0.034		0.0002
U	µg/l	0.38	0.34-0.41	0.39	0.36-0.42	0.97	0.93-1.01	0.98		0.00003
Y	µg/l	0.052	0.048-0.055	0.062	0.050-0.074	0.240	0.222-0.258	0.243		0.0004
Zn	µg/l	1049	1017-1081	845	791-899	1009	896-1120	1294	850-980	1.197

* Range is given as 95% confidence interval

DL = detection limit given as 3 * standard deviation of blank samples

Table 3. Trace element concentrations in serum from controls (n=99) and PD patients (n=33) in samples collected prediagnostically in 1995-97. Odds ratios (OR) and 95% confidence intervals (CI) for the multiple regression analysis are given together with the p-values.

		Controls		**PD patients**		**OR**	**95% CI**	**p-value**
Element	Unit	Average	SD	Average	SD			
As	µg/l	3.7	4.4	2.5	2.3	0.91	0.79-1.04	0.170
Ca	mg/l	103	13	105	11	1.03	0.99-1.07	0.150
Co	µg/l	0.149	0.154	0.118	0.057	0.98	0.93-1.04	0.548
Cr	µg/l	0.75	1.57	0.60	1.26	0.95	0.60-1.50	0.818
Cu	µg/l	1184	293	1119	230	0.97	0.82-1.13	0.662
Fe	µg/l	1146	463	1275	551	1.74	0.73-4.17	0.216
Hg	µg/l	0.48	0.25	0.40	0.15	0.69	0.50-0.97	0.031
Mg	mg/l	21.4	3.0	22.0	3.0	1.17	0.99-1.39	0.068
Mn	µg/l	2.08	1.07	2.09	0.81	1.06	0.66-1.69	0.821
Mo	µg/l	0.91	0.41	1.07	0.43	3.13	0.87-11.25	0.081
Ni	µg/l	0.46	0.66	0.45	0.42	1.48	0.60-3.38	0.397
Pb	µg/l	0.34	0.49	0.29	0.43	0.98	0.88-1.08	0.640
Rb	µg/l	185	36	172.1	27.9	0.99	0.98-1.01	0.355
Se	µg/l	109.6	16.1	111.4	15.0	1.01	0.98-1.04	0.492
Sr	µg/l	28.4	7.6	26.8	6.8	0.99	0.93-1.06	0.819
Tl	µg/l	0.015	0.007	0.013	0.003	0.90	0.80-1.02	0.095
U	µg/l	0.009	0.003	0.014	0.024	1.07	0.91-1.25	0.416
Y	µg/l	0.030	0.013	0.029	0.010	1.01	0.97-1.05	0.577
Zn	µg/l	992	304	994	311	1.00	0.87-1.14	0.969

Table 4. Trace element concentrations in serum from the same patients collected before and after diagnosis with p-values from paired sample t-test. Statistically significant differences are indicated in italics

		Before diagnosis (1995)		**After diagnosis (2007)**		**Paired sample t-test**
Element	Unit	Average	SD	Average	SD	p-value
As	µg/l	3.3	2.7	2.5	3.0	0.269
Ca	mg/l	*104*	*13*	*89*	*9*	*0.001*
Co	µg/l	0.136	0.066	0.292	0.501	0.240
Cr	µg/l	0.99	1.76	1.40	1.29	0.233
Cu	µg/l	1152	282	1116	258	0.497
Fe	µg/l	*1130*	*428*	*949*	*325*	*0.023*
Hg	µg/l	*0.39*	*0.13*	*0.56*	*0.31*	*0.010*
Mg	mg/l	*21.7*	*3.5*	*20.7*	*2.5*	*0.003*
Mn	µg/l	*2.02*	*0.50*	*1.27*	*0.81*	*0.000*
Mo	µg/l	1. 07	0.44	1.01	0.51	0.943
Ni	µg/l	*0.39*	*0.35*	*1.14*	*0.50*	*0.006*
Pb	µg/l	0.34	0.52	0.16	0.40	0.637
Rb	µg/l	*170*	*27*	*153*	*37*	*0.050*
Se	µg/l	*109.8*	*16.9*	*73.0*	*18.3*	*0.000*
Sr	µg/l	25.9	5.3	28.1	12.7	0.101
Tl	µg/l	0.013	0.004	0.013	0.006	0.128
U	µg/l	0.010	0.002	0.022	0.009	0.768
Y	µg/l	*0.031*	*0.013*	*0.098*	*0.039*	*0.000*
Zn	µg/l	1026	323	964	360	0.336