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Radioanalytical and nuclear techniques in trace metal toxicology research

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Abstract

Trace metal toxicology research aimed at generating human-relevant information for risk assessment requires the use of sensitive and sophisticated analytical techniques to determine typically µg/Kg or lower concentrations of trace metals in tissues, cells, intracellular components of laboratory animals and humans. The results of these techniques are needed for an understanding of the biochemical mechanisms and bio-transformations involving trace metals. In this context, radioanalytical and nuclear methods plays a pivotal role. In order to give an idea of typical results which can be obtained by radioanalytical and nuclear techniques when used in combination with biochemical and molecular biology techniques of cellular fractionation we report here some typical studies carried out by means of non-carrier added radiotracers with high specific activity, and neutron activation analysis (NAA). The investigations have been performed in the context of an integrated and complementary *in vivo* and *in vitro* approach that uses both animal and human test systems. Applications reported concern :

(i) the *in vivo* work on laboratory animals (brain regional thallium distribution in rats and identification of thallium binders in testis, by means of $^{201+202}$Tl); (ii) *in vitro* investigations on cells of animal origin (arsenic uptake and biomethylation in rat brain aggregates, neurons, microglia and astrocytes as well as speciation of vanadate in Balb/3T3 cells, by means of ^{73}As and ^{48}V, respectively); *in vitro* experiments on cell of human origin (intracellular behavior of cadmium in human umbilical cord blood (UCB) stem cells, by means of ^{109}Cd); analytical determinations of trace metals in tissues of general population and patients potentially affected by metal-related disease, by means of NAA.

The analytical determinations carried out allowed to relate total element concentrations in cells to the results of investigations at the intracellular and molecular levels with the goal of identifying the biochemical components that interact with trace metals. These s findings demonstrate the great potential of radioanalytical and nuclear techniques in the context of an integrated *in vivo*-*in vitro* strategy adopted in trace metal toxicology research for a mechanistically-based hazard characterization concerning the exposure to low doses of trace metals.

Keywords

Radiotracers, neutron activation analysis, cell cultures, trace metals, metal toxicology

Introduction

Trace elements, oligoelements, trace metals, heavy metals, metals. Although over time the literature concerning the understanding of the biological role of chemical elements in human life has accepted these terms as “normal” each of them has become synonymous of different fields of interest, i.e. concerning nutritional, environmental, occupational and biomedical exposure to chemical elements. In this context, although all chemical elements are toxic in certain forms and in sufficiently high doses “heavy metals” are

generally accepted as synonymous with pollution and toxicity. Here we adopt the term "trace metal" that reflects the toxicological character of the applications presented.

The term trace metals is not related to man-made but it is their re-distribution in nature that has strongly altered the human exposure. They occur in nature, are not destroyed and circulated in natural cycles through the environment, usually at ppm levels or less [1]. In the modern era the broad scale of environmental changes, mineral pollution of air, water and soil connected with the growth of industry, urbanization, development of transport, the use of agrochemicals and changes affecting human and animal nutrition have induced profound changes in the exposure of large population groups to certain trace metals overgrowing the limits of geochemically or professionally exposure. As this represents a potential risk for public health the EU Chemical Policy foresees regulatory actions to prevent health risks of exposure to certain metals [2–7]. Although the setting of threshold limits of trace metal exposure is a matter of policy decision their basis is essentially a scientific task. The toxicological assessment of the risk to humans, associated with exposure to trace metals, involves the integration of the results of three steps: hazard identification, dose-response assessment and exposure assessment. In this context, the conventional approach used for hazard characterization, principally based on toxicological data from animal experimentation, is an approach considered defective in the assessment of risks to humans [8]. In addition, in the last decades the use of live animals in experimental studies has raised ethical questions. However, the spectacular development of new cellular, molecular and biochemical tools provides the opportunity to obtain mechanistic information and play important role in studying metabolic patterns and toxicity of trace metals at cellular and molecular level, significantly improving the scientific basis of risk assessment [9]. At present, the integration of *in vivo* and *in vitro* data from animals and human test systems ("parallelogram approach", Fig. 1) is of the best approach in trace metal toxicology research aimed at generating human-relevant information for risk assessment [10].

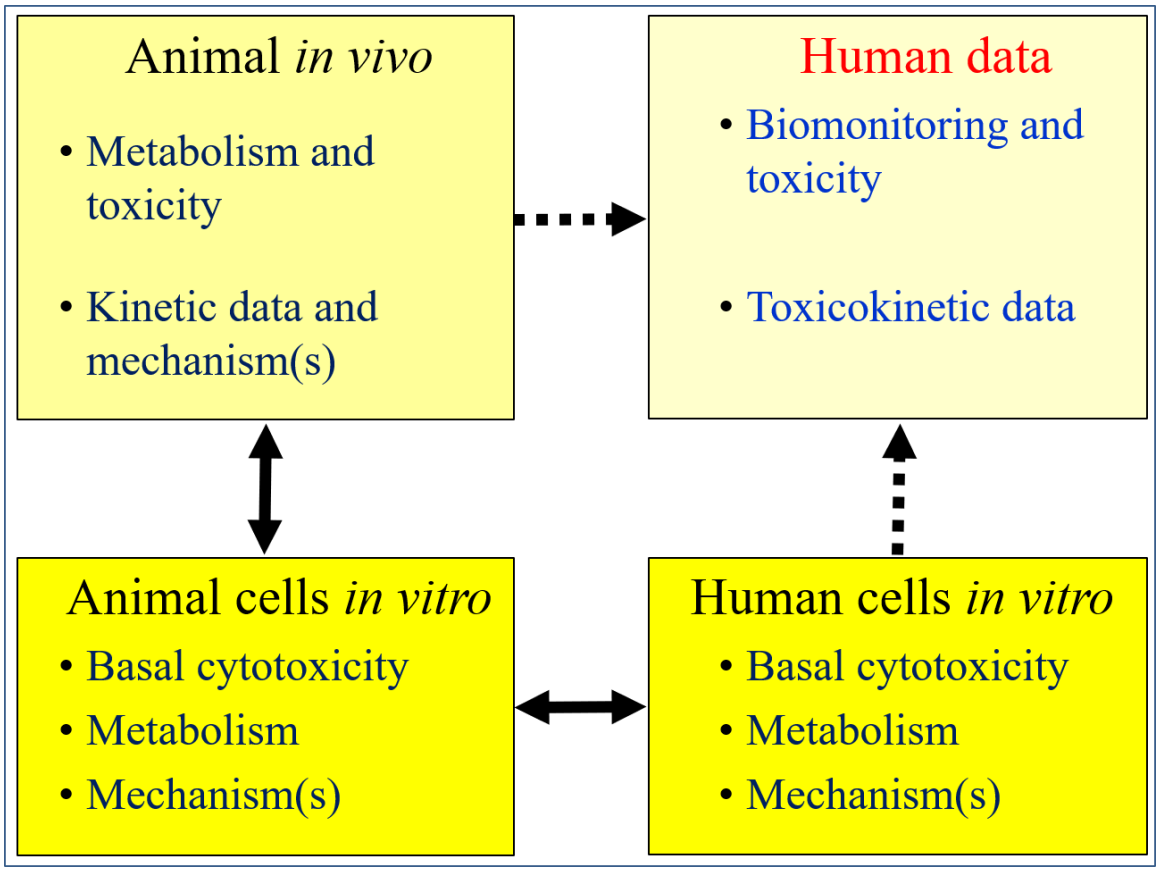

Fig. 1 The parallelogram approach [10] adopted in trace metal toxicology research. It involves the use of *in vivo* and *in vitro* data as part of an integrated strategy for hazard assessment and human risk assessment of trace metals. The *in vitro* systems are employed to obtain information on mechanisms of metal toxicity as well as to investigate the relevance of animal response to humans. Solid lines: data comparison. Dotted lines: extrapolations where no appropriate *in vivo* human data are available

There are some areas of research priorities with regard the relationship of trace metals to diseases: (i) toxicity due to excess exposure and tissue accumulation of a metal; (ii) biochemical mechanisms of trace metal toxicity; (iii) changes occurring in trace metal concentrations in tissues/body fluids to be used in diagnosis and treatments of diseases.

The risk that large population groups can be exposed to trace metals in a form and dose incompatible with good health with the induction of toxicity at low level of exposure has opened new analytical problems in trace metal toxicology research. In highly developed

countries the quantitative differences between environment and occupational exposure shifted from "high to low" dose exposure [11]. Consequently, interest in the analysis of trace metals in biological samples has shifted from concentrations of mg/kg or greater for some metals such as Cu and Zn to ng/kg and lower concentrations for toxic metals such as As, Cd, Hg, Pb, Sb, Tl and V. The study of the metabolic pathways of trace metals and the biochemical mechanisms responsible for their retention and toxic effects is essential for the evaluation of dose-effect relationships. These investigations require knowledge of the distribution of trace metals in the cellular compartments of different tissues and biological fluids, and protein fractions which are obtained after long and complex separation procedures. Thus, very sensitive analytical techniques for ultra-micro determination of trace metals in biological samples at µg-ng/kg in combination with bioanalytical techniques of cellular fractionation are necessary to carry out metal toxicology research at molecular level [12].

In addition, the chemical form (i.e., species) of trace metals can influence their toxicokinetics and should be considered to improve human health risk assessment. The determination of "total" trace metal concentrations in tissues, subcellular and molecular components is no longer sufficient and must be followed by metallomic studies (determination of metal species within a cell or tissue type and their interactions, transformations, and functions in biological systems [13] that become increasingly important in regulatory trace metal toxicology.

The aim of this paper is to highlight the high degree of applicability that nuclear and radiochemical techniques (neutron activation analysis (NAA) and use of radiotracers with high specific activity) have in trace metal toxicology research at tissue, intracellular and molecular level. Original applications are presented as examples of scientific data that can be obtained from studies carried out in the context of the parallelogram approach (Fig.1), i.e. from *in vivo* experimental models (laboratory animals), *in vitro* with animal and human test systems (cell cultures) and studies on differently exposed humans.

Experimental

Chemicals and biochemicals

Sodium metavanadate(V) ($NaVO_3$, CAS 13718-26-8), vanadium(IV)oxide sulfate pentahydrate ($VOSO_4x5H_2O$, CAS 123334-20-3),sodium(meta)arsenite(III) ($NaAsO_2$, CAS 7784-46-5), thallium sulphate (Tl2SO4 ,CAS 7446-18-6) were supplied by Sigma Aldrich (Milan, Italy); $CdCl_2$ (CAS 10108-64-2) by Alpha Aesar (Karlsruhe, Germany)

Radioisotopes and radioactive counting

^{73}As ($T_{1/2}$= 80.3d) in 0.1 M HCl was purchased by Los Alamos National Laboratory (Los Alamos, USA) (specific activity 1.6 mCi/µg As, radiochemical purity >99.5%)

The following non- carrier added (NCA) radiotracers were produced at the JRC-Scanditronix MC40 cyclotron and prepared for biochemical use as previously described: ^{109}Cd ($T_{1/2}$= 462d), by proton irradiation of natural Ag targets (specific activity:1 µCi/ng Cd) [14]; $^{201+202}Tl$ ($T_{1/2}$= 3.04 and 12.2d, respectively), by proton irradiations of metallic Hg target (specific activity: 48 µCi/ng and 5 µCi/ng of Tl, respectively) [15]; ^{48}V ($T_{1/2}$= 16.1 d), by (α,n) nuclear reaction on metallic Sc foil (specific activity: 1.3 µCi/ng of V) [16].

In experiments with individual radiotracers the counting of the radioactivity in animal tissues, cell culture systems, their subcellular components and chromatographic fractions from size exclusion chromatography (SEC) was carried out by integral γ-counting with a Wizard 3 Gamma Counter (Perkin Elmer, Life Sciences) apparatus equipped with a well-type 3.15" x 3" NaI(Tl) size crystal [17]). For each radioisotope appropriate energy threshold and windows were set, depending on the characteristic line photon emissions. For radiotracers that were not in the radioisotope library for automatic calibration (^{73}As, ^{202}Tl, ^{48}V) a manual calibration was carried out. In this context, sample of the radiotracer solutions were placed in the counter to obtain the gamma ray spectra. From the spectra and for the main gamma peaks the lower and upper energy levels were determined. These values were used to set appropriate energy threshold and windows for our experimental conditions. Each time radioactivity measurements have been interpreted in terms of

exogenous element concentration by comparing them with radioisotopes standard reference solutions of known specific radioactivity.

Neutron activation analysis

Neutron activation analysis of Cr and Sb in human tissues, body and dialysis fluids has been carried out as previously described. [18–21]. Briefly, 0.5–1g of tissue sample or 1 mL of freeze-dried biological fluid in sealed quartz or plastic vials were irradiated for 24 h in the HFR nuclear reactor (Petten, The Netherlands) in a thermal neutron flux of $2x10^{14}$ neutrons $cm^{-2}\,s^{-1}$. One week later ^{51}Cr and $^{122+124}Sb$ were radiochemically separated from irradiated samples [21] and the characteristic γ ray emissions (^{51}Cr: directly counted by computer–based ray spectrometry (INAA) using a HPGe detector (EG&G Ortec Int, GA, USA) coupled to a Laben 70 multichannel analyser (Laben, USA) equipped with automatic samples changer. The acquisition and analysis of the spectra has been carried out by specific software (Nuclear Elements Digital Analysis, NEDA, Ascom, Milano) [22].

***In vivo* studies by experimental animal models**

Brain regional and testis $^{201+202}Tl(I)$ distribution in rats

Animals used were Male Sprague Dawley rats (230-250 g, Harlan Nossan, Correzzana. Italy. They were maintained with commercial food in pellets and natural mineral water (Acqua Panna, Tuscania, Italy) ad libitum. Housing conditions and experimental procedures were in strict accordance with the European Community regulations [23]. The animals were acclimated to the housing conditions for at least 2 days before intraperitoneal (i.p.) administration of 10 ng $^{201+202}Tl(I)$ /rat. At 24 h post-injection (testis distribution) or at times from 4 h to 57 d (brain regional distribution) animals were sacrificed by cardiac puncture under ether anesthesia. Tissues were removed, weighed, washed with 0.9% saline solution and analyzed for the radioactive content. Blood was collected by heparinized syringe and centrifuged to separate red blood cells and plasma. Brain regions (cortex, corpus striatum, cerebellum, hippocampus, hypothalamus and medulla oblongata) were dissected according Glowinski and Iversen, [24] Liver and testis

were homogenized in sucrose–cacodylate medium and submitted to differential centrifugation to isolate cellular organelles and cytosol (TL-100 ultracentrifuge, Beckmann Instrument, Palo Alto, CA, USA). Tissues, brain regions and subcellular fractions were counted for the $^{201+202}$Tl content [25]. Size exclusion chromatography (SEC) of testis $^{201+202}$Tl-cytosol and plasma as well as ultrafiltration experiments were carried out as previously described [17].

In vitro studies on cells of animal origin

^{73}As(III) in rat brain aggregates, neurons, microglia and astrocytes

Brain re-aggregating cultures were prepared from 16-day old fetal rat telencephalon at the Insubria University (Varese, Italy) according to Honegger and Monnet-Tschudi [26]. After re-suspension in Dulbecco's Modified Eagle's medium (DMEM) high Glucose culture medium (7.5·106 cells/mL) cells were maintained at 37°C in an atmosphere of 10% CO_2.

Primary cerebellar granule cells (neurons), mixed primary glial cells containing 85% of astrocytes and microglial cells were isolated and cultured as reported elsewhere [27–28]

Uptake studies of ^{73}As by aggregates or individual neurons, astrocytes and microglia cells were carried out by exposing cell cultures to 0.1–1000 µM (aggregates) or 0.1–30 µM of ^{73}As(III). The aggregates were counted by microscope and the incorporated 73As counted by integral γ counting. Proteins were determined by the Lowry protein assay [29]. Cell viability of the individual type of brain cells has been determined by tetrazolium salt, 3-(4,5 dimethylthiazol-2-yl)-2-5 diphenyltetrazolium bromide (MTT) assay [30].

Biomethylation study. Brain aggregates, neurons, microglia and astrocytes exposed to ^{73}As(III) were resuspended, homogenized, ultracentrifuged and the extract submitted to ion exchange chromatography on AG 50-X4 resin for the separation of the inorganic and biomethylated As species [31]. These species were also measured in the culture medium at the end of the experiment. ^{73}As in the fractions containing the ^{73}As species were counted by integral γ counting.

211 Speciation study of vanadate in Balb/3T3 cells

212 The cellular line of immortalized mouse fibroblasts Balb/3T3 (clone A31-1-1) has been
213 provided by the Istituto Zooprofilattico Sperimentale of Lombardia and Emilia (IZS),
214 Laboratorio Centro Substrati of Brescia. They were maintained in culture and cultured as
215 previously described [32]. Balb/3T3 cells were exposed for 72 h to non-transforming
216 (0.1 µM) and transforming (10 µM) concentrations of pentavalent $^{48}VO^{-3}$ species as well
217 as to the same concentrations of the non-transforming [33] tetravalent $^{48}VO_2^+$ ions. Then,
218 the uptake, intracellular distribution of ^{48}V as well as the SEC profiles of the ^{48}V-
219 containing cytosols were determined as previously reported [33].

220 The determination of the oxidation state of vanadium of the ^{48}V-containing peaks
221 emerging in the eluate from SEC was carried out by chromatography on Chelex 100 resin
222 (Fig. 2).

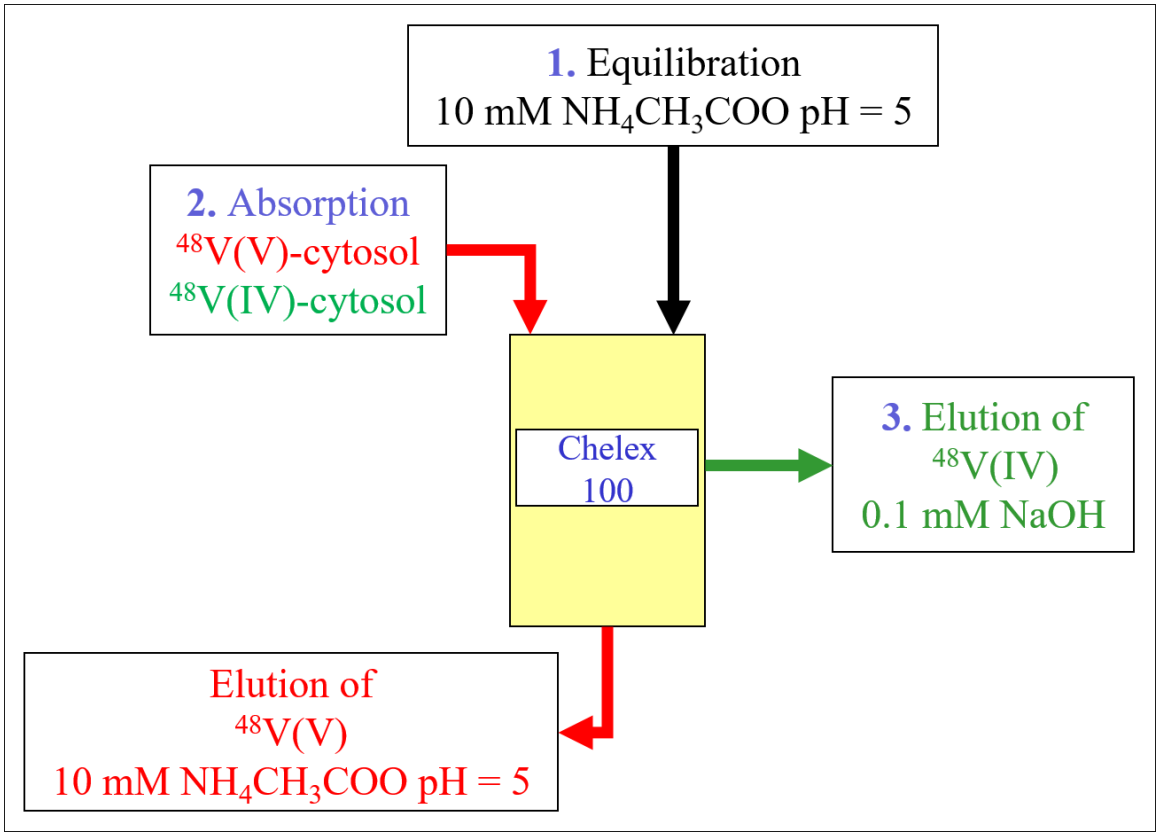

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Fig. 2 Method of speciation of vanadium in Balb/3T3 cells exposed to pentavanadate or tetravanadate species

The binding of vanadium to DNA has been investigated by: (i) isolating highly pure DNA from cells exposed to 5 μM of ^{48}V (V); (b) incubating the DNA previously isolated from unexposed cells with 5 μM of ^{48}V (V). In both cases the ^{48}V-DNA fraction isolated from the cells or the incubation mixture ^{48}V-DNA were submitted to SEC on Sephadex G25 recording UV and ^{48}V profiles in the eluate.

***In vitro* studies on cells of human origin**

Intracellular behavior of cadmium in ^{109}Cd(II)–exposed human umbilical cord blood (UCB) stem cells

Human haematopoietic CD34+ progenitor cells were purified from umbilical cord blood (UCB), cultured and ex-vivo expanded as previously described [34]. UBC samples (20–60 mL) were collected with informed consent of donors, from full-term deliveries at the Department of Gynaecology and Obstetrics, SS. Annunziata Hospital, Chieti.

Uptake and intracellular distribution of cadmium were studied by exposing UCB cells for 72 h to concentrations of ^{109}Cd (II) ranging from 0.1 to 10 μM [34]. The distribution pattern of cadmium in the ^{109}Cd-cytosol obtained by ultracentrifugation from cell homogenate was studied by Fast Protein Liquid Chromatography (FPLC) (Pharmacia, Sweden) using a Sephacryl S200 column, followed by record of the UV and integral γ counting of ^{109}Cd in the eluate.

Studies on humans

Trace metal reference values in general population

The population sampled consisted of inhabitants living in of the provinces of Messina, Catania and Palermo of Sicily region, south Italy. Subjects selected (n= 325, mean age 43.7 y, range 23-62 y, 60,7 % males, 39.3% females) were representative of eight sub-groups residing in sites that reflect different environmental characteristics of the Sicilian region. The Institutes of Occupational Medicine and Preventive Medicine of Messina,

Catania and Palermo collected blood, urine and hair samples according to previously developed protocols [21]. In this context, antimony was determined in such specimens by radiochemical separation NAA, involving absorption on neutron activated mineralized sample on tin dioxide (TDO), followed by γ ray spectrometry [21]. In order to form the best possible homogeneous group only basic requirements of acceptance were non-smokers, and not to have been professionally exposed to Sb during their lifetime.

Chromium overload in dialysis patients

The study was undertaken in patients on chronic dialysis at the Nephrology Unit of the San Giovanni Molinette Hospital, Turin (serum and tissue analysis) and at the Nephrology Unit of the S.Paolo Hospital, Milan (intradialytic mass balance). All patients were consenting and informed on the objectives of the study. The study design was approved by the institutional ethics committees. Autopsies were performed in accordance with the principles of the Declaration of Helsinki. Tissues samples were collected at autopsy from 18 uremic patients on chronic dialytic treatment (10 males, 8 females, age 51 ± 16 y, range 32–79, dialytic age 123 ± 62 months). Autopsy specimens from 16 subjects without renal diseases and with age-matched patients were used as controls. *In vivo* intradialytic Cr mass balance during standard bicarbonate dialysis was evaluated in 8 uremic patients under standard bicarbonate dialysis lasting 4 h, using a dialyzer with cuprophan membrane. Blood and dialysate samples were collected at the beginning and end of dialysis together with the total (120 L) 4h outflow dialysate.

Dialysis fluid and autopsy specimens were analyzed by radiochemical separation NAA as reported elsewhere [18, 21]. In intradialytic experiments Cr in whole blood was determined by GFAAS [20].

Applications

In vivo studies by experimental animal models

Brain regional and testis $^{201+202}$Tl(I) distribution in rats

Thallium is an extremely high toxic element. In humans thallium poisoning is mainly due to acute intoxication. It is not surprising that the bulk of the literature on its metabolism and toxic effects in experimental animal models concern acute/subacute exposure of the animals. However, thallium has acquired increasing importance as a chemical pollutant due to the consumption of contaminated food (vegetables, fish, meat-based products) and drinking water [35]. In spite of this, very few *in vivo* studies on experimental models are available on metabolism and toxicokinetics of environmental levels of the element [36]. Here we present two studies on metabolic patterns of low doses (ng level) of thallium in male rat reproductive systems and brain, two target tissues for thallium reproductive toxicity [37] and neurotoxicity [38].

About the neurotoxicity of thallium is related to its preferential effects in some brain regions [39], being the differential accumulation of thallium in brain topography a possible explanation. This hypothesis was verified in experimental animals only for acute exposure (i.p.injection of tens of mg/kg b.w. [40], millions of times greater than typical human environmental exposure (estimated human daily dietary intake: 2 ng Tl g^{-1}) [35]. In this context, small but significant amounts of the element was detected in the rat brain at 8 d after i.p.injection of environmental dose (2µg Tl/rat) [41].However, this latter study was carried out with ^{201}Tl radiotracer whose half life ($T_{1/2}$=3.04d) not allowed to examine metabolic fate and persistence of the element in the organ for longer period. For this, we optimized the radiolabelling of nanograms of Tl+ ions with the radiotracer ^{202}Tl whose half-life ($T_{1/2}$= 12.2d) has made possible to follow the metabolic fate of thallium in brain areas for up to 57 days.

Table 1 Time course of thallium in brain regions. Rats were i.p.injected with 10 ng $^{201+202}$Tl(I)/rat and sacrificed at the times indicated

	Tl content (pg g^{-1} wet w)[a]					
	4 h	**12 h**	**1 d**	**10 d**	**28 d**	**57 d**

Cortex	1.3 ± 0.2	1.5 ± 0.2	2.4 ± 0.5	1.1 ± 0.2	1.1 ± 0.4	0.9 ± 0.2
Corpus striatum	1.5 ± 0.2	1.7 ± 0.2	2.9 ± 0.3	1.2 ± 0.2	1.0 ± 0.3	0.8 ± 0.3
Cerebellum	1.4 ± 0.3	1.6 ± 0.3	2.8 ± 0.4	1.3 ± 0.3	0.9 ± 0.5	0.8 ± 0.2
Hyppocampus	1.5 ± 0.4	1.9 ± 0.4	2.8 ± 0.5	1.6 ± 0.4	1.4 ± 0.4	1.2 ± 0.4
Hypothalamus	2.2 ± 0.4	2.7 ± 0.4	4.2 ± 0.2	2.8 ± 0.4	2.0 ± 0.8	1.7 ± 0.2
Medulla obl.	1.6 ± 0.3	1.7 ± 0.3	3.2 ± 0.4	1.7 ± 0.3	1.8 ± 0.4	1.0 ± 0.3

a: mean value of 4 animals ± SD

Table 1 summarizes the brain regional thallium distribution in rats i.p.injected with 10 ng ^{202}Tl(I)/rat. The time course of thallium distribution in the brain regions was similar, the hypothalamus having consistently the highest thallium concentrations. A maximum of ^{202}Tl in all brain regions was achieved 1d after injection, approximately halving after 10d, and then declining less rapidly up to 57 days.

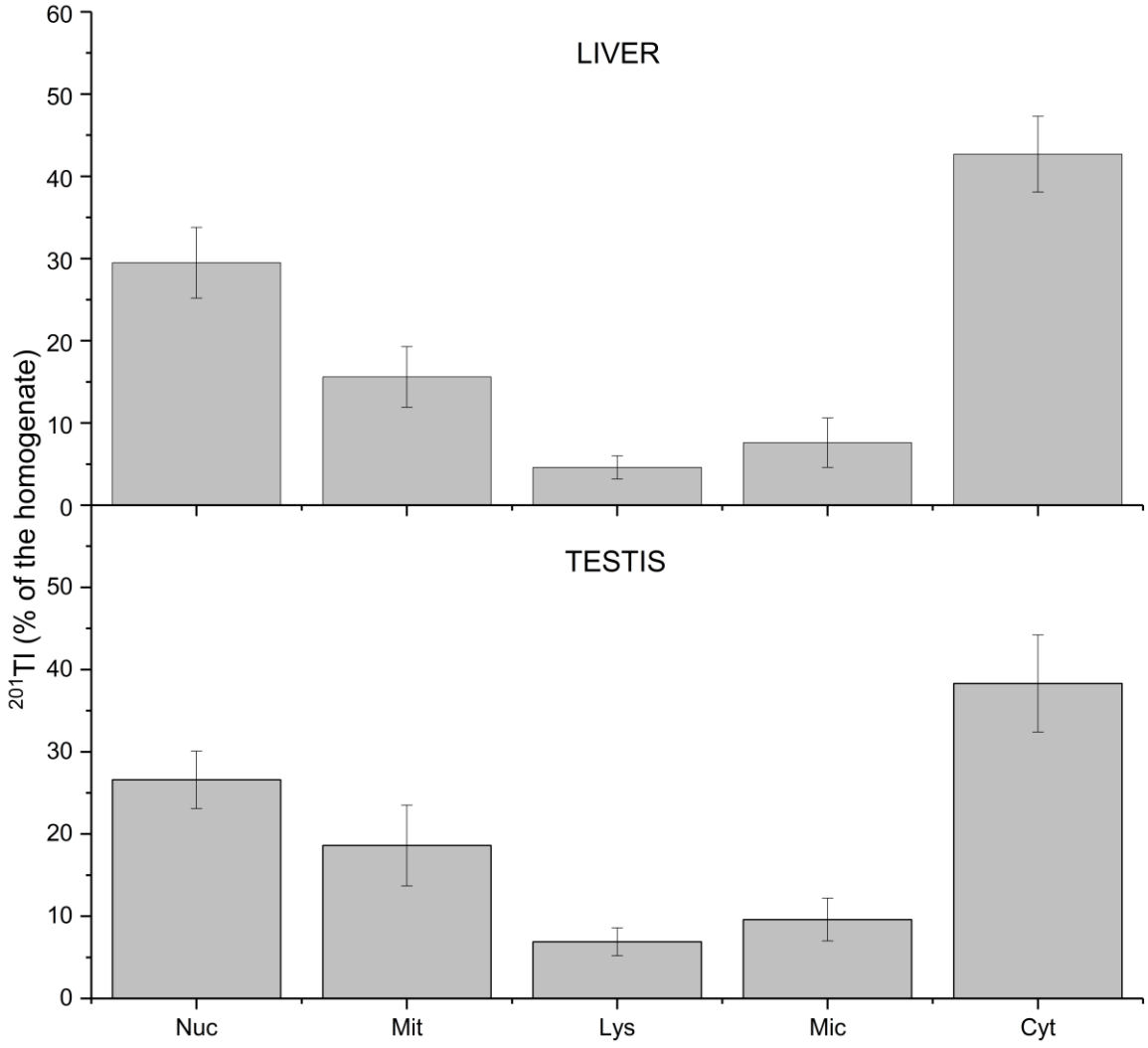

Fig. 3 Intracellular distribution of ^{201}Tl in testis of rats 24h after i.p. injection of 10 ng ^{201}Tl/rat, as chloride. The subcellular fractions were isolated by differential centrifugation and counted for the ^{201}Tl content. The results, expressed as the percentage of the ^{201}Tl in the homogenate, are the mean of individual tissues of 4 rats. Nuc(nuclear), Mit (mitochondrial), Lys (lysosomal); Mic (microsomal), Cyt (cytosol) fractions

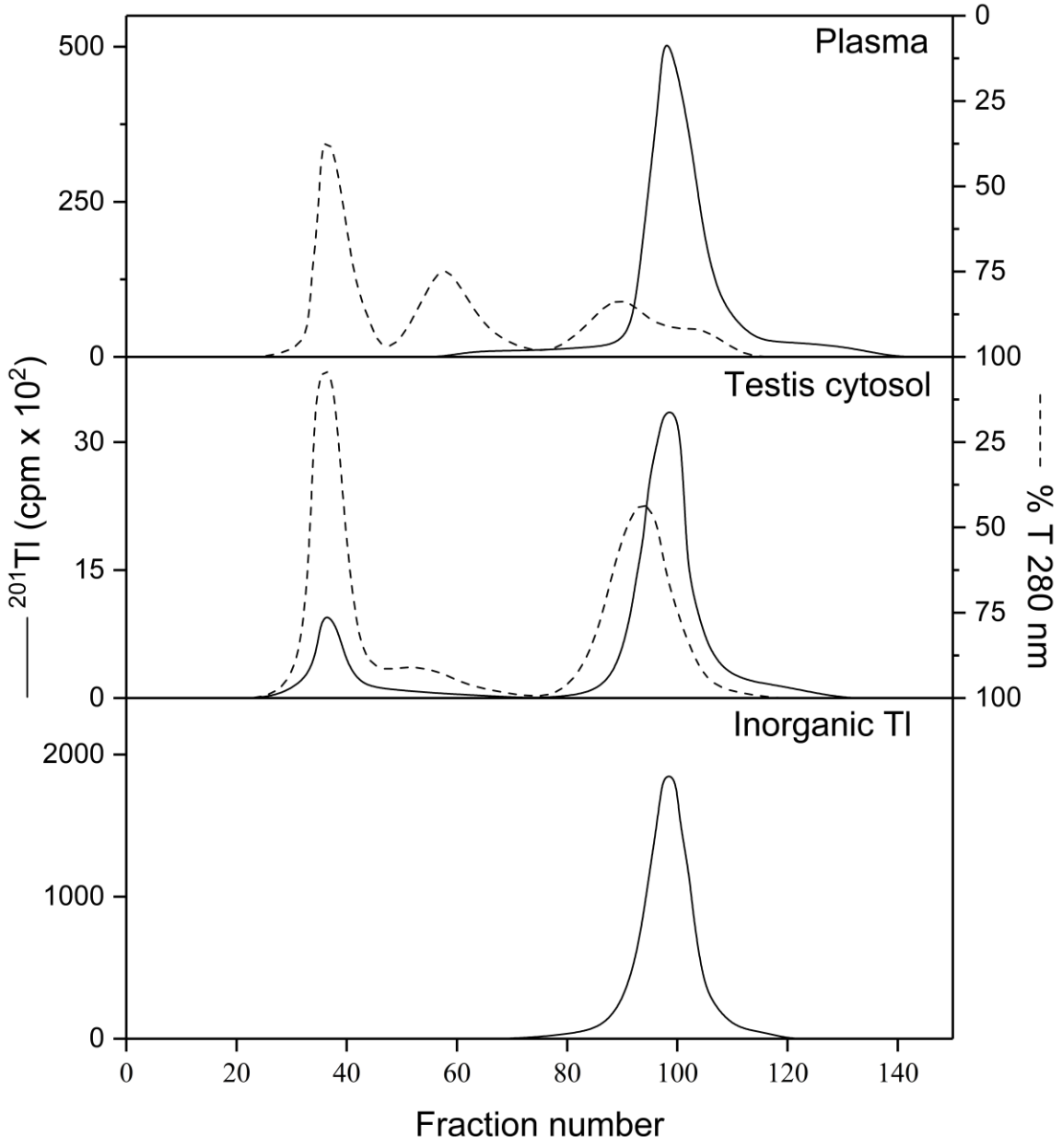

Fig. 4 ^{201}Tl elution patterns of Sephadex G-150 (2.5x110cm) fractionated rat testis cytosol 24 h after i.p. injection of 10 ng ^{201}Tl (I) per /rat. Elution was carried out at 4°C with 10mM cacodilate-HCl buffer, pH=7.2. The column was previously calibrated with a kit of biochemical of known molecular weight [17]

About the interaction of thallium with testis, 24 h after i.p. injection of 10ng ^{201}Tl(I)/rat the testis contained 0.85% of the dose, mainly recovered in the cytosol followed by nuclear and mitochondrial fractions as observed in the liver (Fig. 3). Blood contained

0.04% of the dose mL^{-1}, 35% in the plasma and 66% in RBC. SEC of the ^{201}Tl- cytosol shows two peaks of radioactivity (Fig. 4): the first ^{201}Tl-peak I (approx. 20% of the cytosolic thallium) immediately after the void volume in which ^{201}Tl was firmly bound to high molecular weight components (HMWCs). The second main ^{201}Tl-peak II emerged in fractions corresponding to UV-absorbing low molecular weight components (LMWCs) in position of the ^{201}Tl alone. We were not able to establish if thallium was present as "free" ions or in association with LMWCs.

In vitro **studies on cells of animal origin**

^{73}As(III) in rat brain aggregates, neurons, microglia and astrocytes

Inorganic arsenic exists in a trivalent or pentavalent oxidation state. After intake, inorganic species are biomethylated resulting in organic trivalent and pentavalent arsenic compounds that affect the tissue distribution and retention of arsenic and its toxic effects as a human toxicant [42] Arsenic is considered a human neurotoxin, inducing peripheral neuropathy [43]. However, the mechanisms of arsenic toxicity in living cells of nervous system are not fully understood. In this context, we have investigate the interaction of inorganic As(III) with rat brain aggregate cell cultures and their individual cell components (neurons, microglia and astrocytes). Rat brain aggregates are a model for *in vitro* studies of neurotoxcity of chemicals, including heavy metals [44]. This cell-based toxicity testing is a primary 3D cell culture system (Fig. 5) that contains all the different brain cell types, and preserves cytoarchitecture, circuitry and other biochemical processes [45].

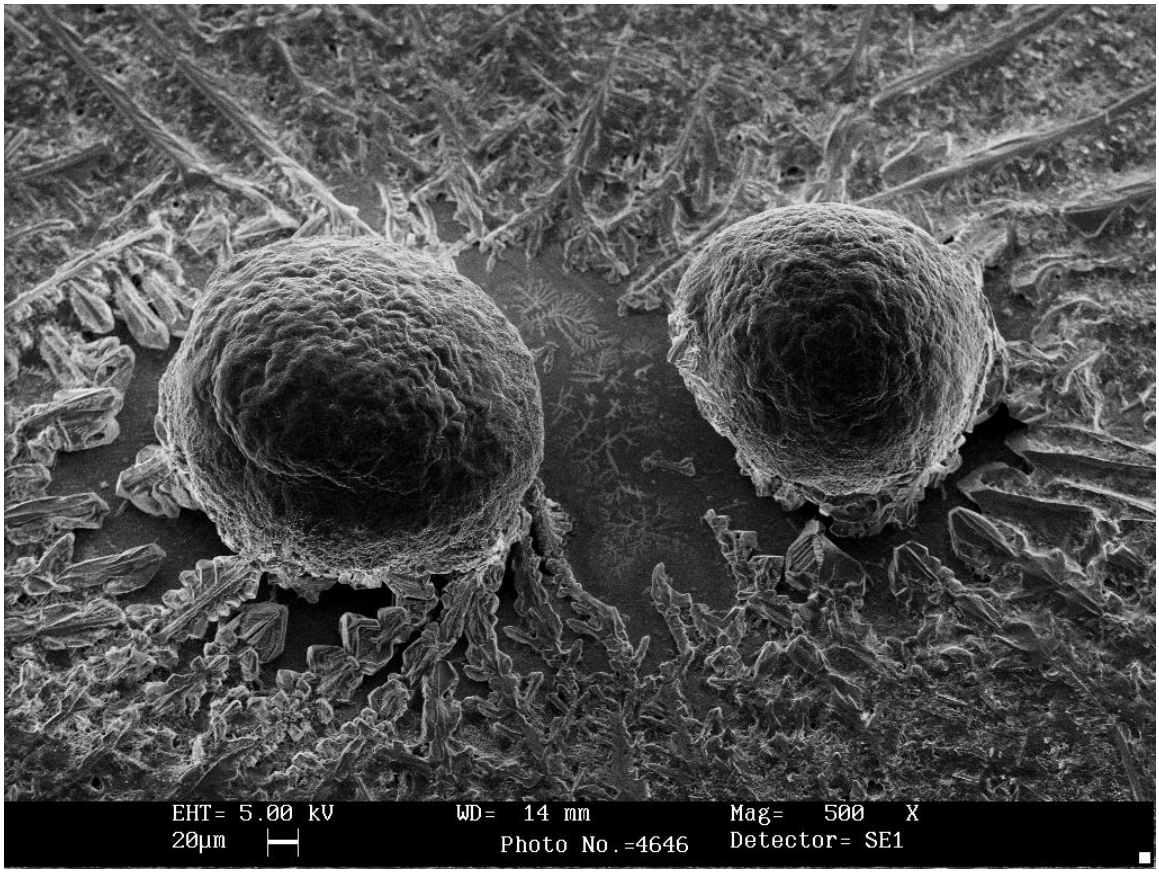

Fig. 5 Brain aggregates microphotography by Scanning Electron Microscope prepared from 16-day old fetal rat telencephalon at the Insubria University (Varese, Italy) according to the protocol of Honegger and Monnet-Tschudi [26]

Table 2 Arsenic uptake in brain aggregates of rats exposed for 72h to inorganic $^{73}As(III)$[a]

Dose (µM)	ngAs/µg proteins	ngAs/aggregate
0.1	0.003	2.6
1	0.010	2.5
30	0.020	5.0
100	0.056	1.4
330	0.053	1.3
1000	0.051	1.2

a: mean of 3 determinations, RSD<15%. Basal value: 0.001 ngAs/µg proteins

Table 2 shows the results of the uptake of ^{73}As in rat brain aggregates. The highest incorporation of As (5ng As/aggregate) was observed at 30 µM. At 100µM this value decreased to 1.4ng As/aggregate which did not significantly changed at higher doses. Inorganic As species, biomethylated forms (monomethylarsonic (MMA(V) and dimethylarsinic (DMA(V) acid) were identified in the extract of brain aggregates and in culture medium at the end of the experiment (Table 3). Overall, inorganic arsenic (III) was able to penetrate the whole rat brain re-aggregates with the formation of biomethylated organic species.

Table 3 Relative percentage of the chemical species of As in the extract of brain aggregates and in complete culture medium after exposure for 72h to inorganic $^{73}As(III)$

Dose (µM)	Aggregate (% of extract)			Culture medium (% of dose)			
	As_i[a]	MMA(V)	DMA(V)	As_i[a]	MMA(V)	DMA(V)	Others
0.1	41	2	57	99.3	0.14	0.49	0.11
30	35	48	17	99.3	0.09	0.03	0.57

a: As_i = inorganic arsenic; MMA = monomethylarsonic acid; DMA=dimethyl arsenic acid

Table 4 Uptake of As by astrocytes, neurons and microglia after exposure for 72h to inorganic $^{73}As(III)$

	pmolAs/10^6cells/h[a]		
Dose (µM)	Astrocytes	Neurons	Microglia
0.1	0.39	0.28	0.06
1	6.19	2.20	0.37
3	11.46	4.44	1.72
10	33.15	8.00	2.65
30	57.13	29.24	11.72

a: mean of 3 determinations, RSD<10%

The results of the uptake of ^{73}As in individual cell components of brain aggregates (neurons, microglia and astrocytes) are reported in Table 4. The incorporation of arsenic into the three types of cells was dose and cell-type dependent

(astrocytes>neurons>microglia). Interestingly, cell vitality of microglial cells was not significantly affected by arsenic. On the contrary, an obvious effect was observed in case of neurons and astrocytes whose the vitality was completely inhibited at 30µM while the microglia survived (Fig. 6).

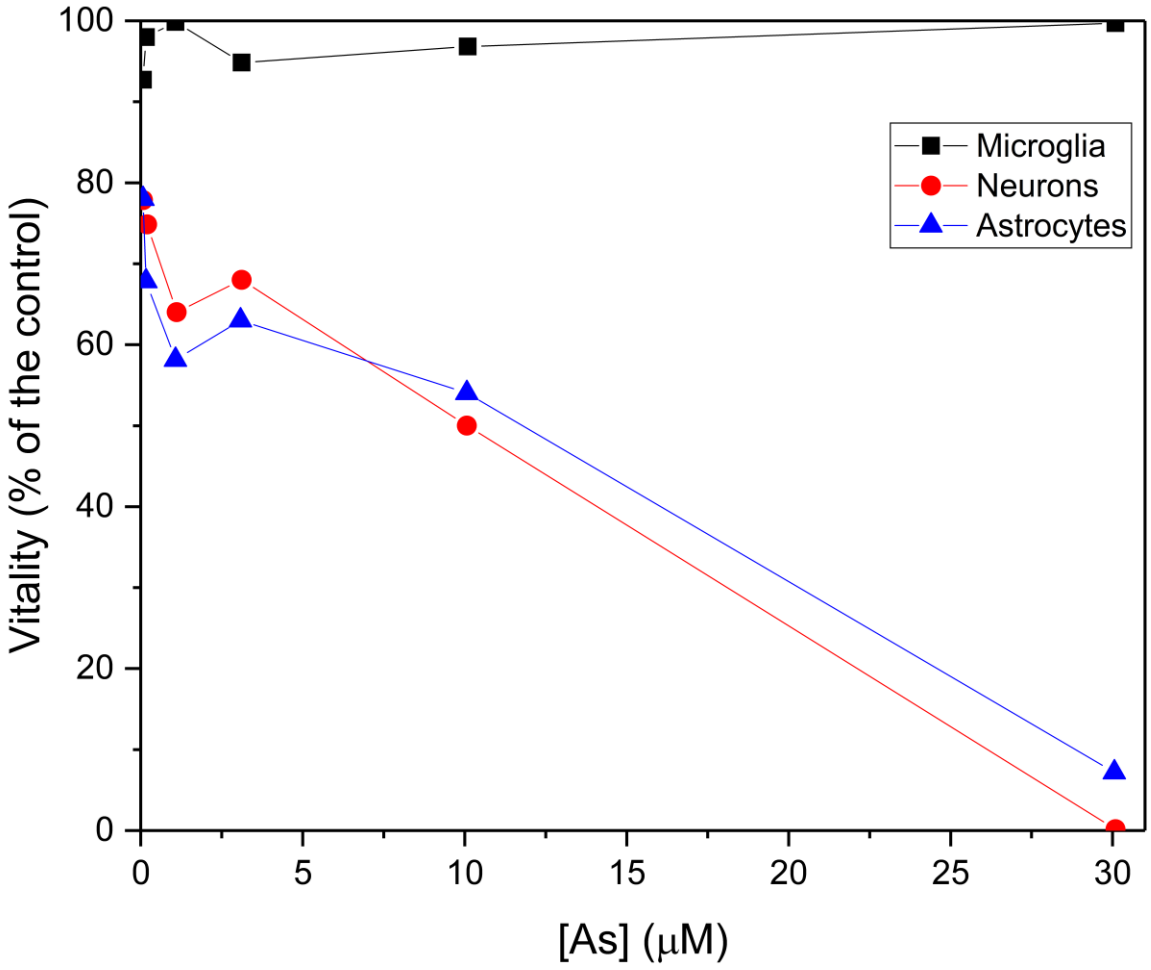

Fig. 6 Cell vitality of microglia, neurons and astrocytes exposed to ^{73}As(III) for 72h as measured using the tetrazolium salt, 3-(4,5 dimethylthiazol-2-yl)-2-5 diphenyltetrazolium bromide (MTT) assay [30]

Speciation study of vanadate in Balb/3T3 cells. Balb/3T3 cell line of mesenchymal origin is one of the most valuable short term test for *in vitro* bioassays allowing a quantitative dose-response to metal carcinogens [46]. Previous *in vitro* study on cultured Balb/3T3 cells has shown a carcinogenic potential of vanadium salts which depends on the oxidation state of the element [47]. While 10 µM pentavalent vanadium induced

morphological neoplastic transformation tetravalent vanadium was proved non-toxic and non-transforming [47]. The key point in determining the intensity of the neoplastic action of V(V) is its intracellular bioreduction to less toxic V(IV) that is considered the detoxication mechanism for the pentavalent vanadium species [48]. In this context, we have undertaken speciation studies (oxidation state of cytosolic vanadium and binding to DNA) to gain new information about the mode of action concerning the neoplastic morphological transformation induced by V(V) in Balb/3T3 cells.

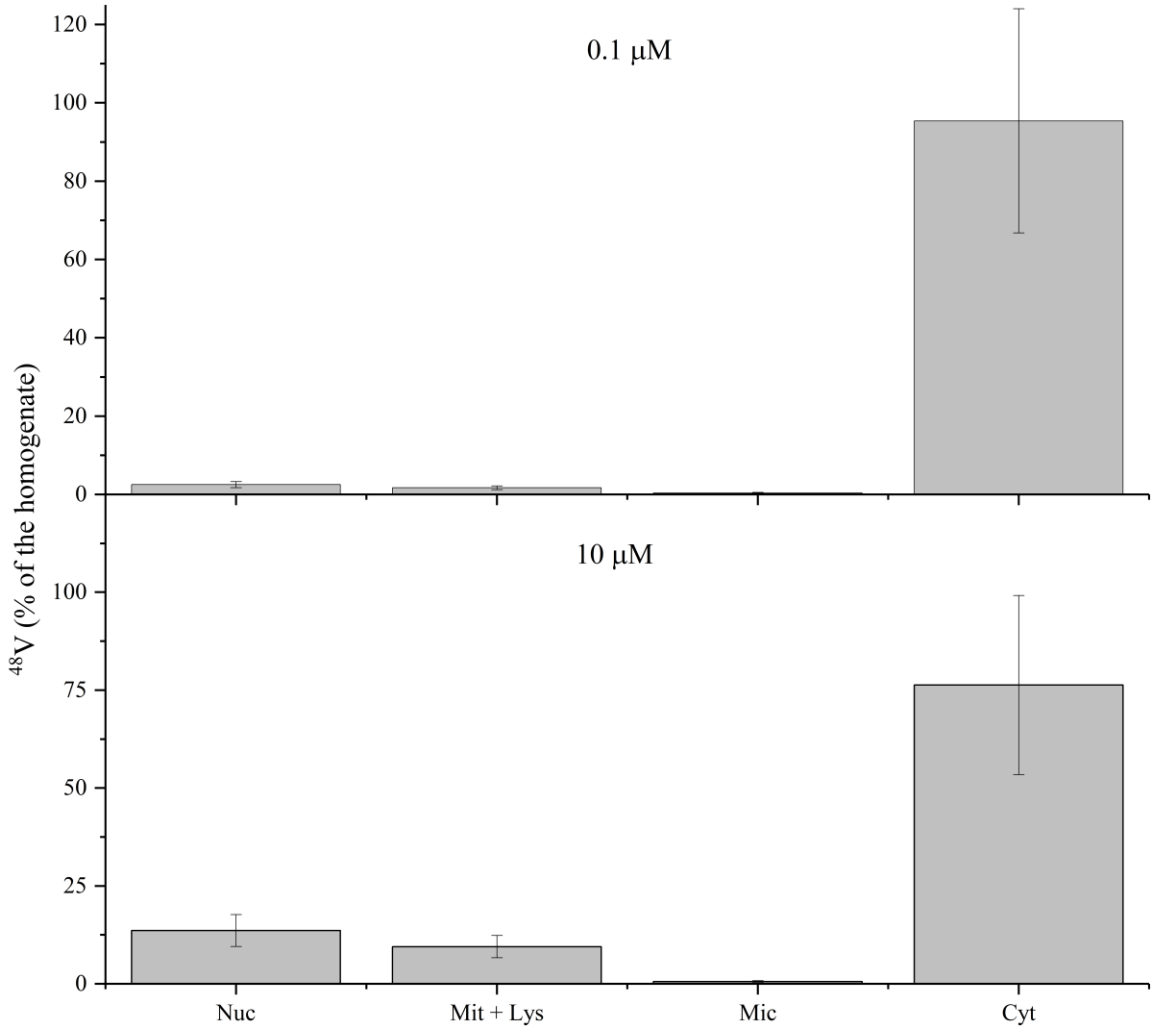

Fig. 7 Intracellular distribution of ^{48}V in Balb/3T3 cells exposed to 0.1 and 10 μM of ^{48}V(V)

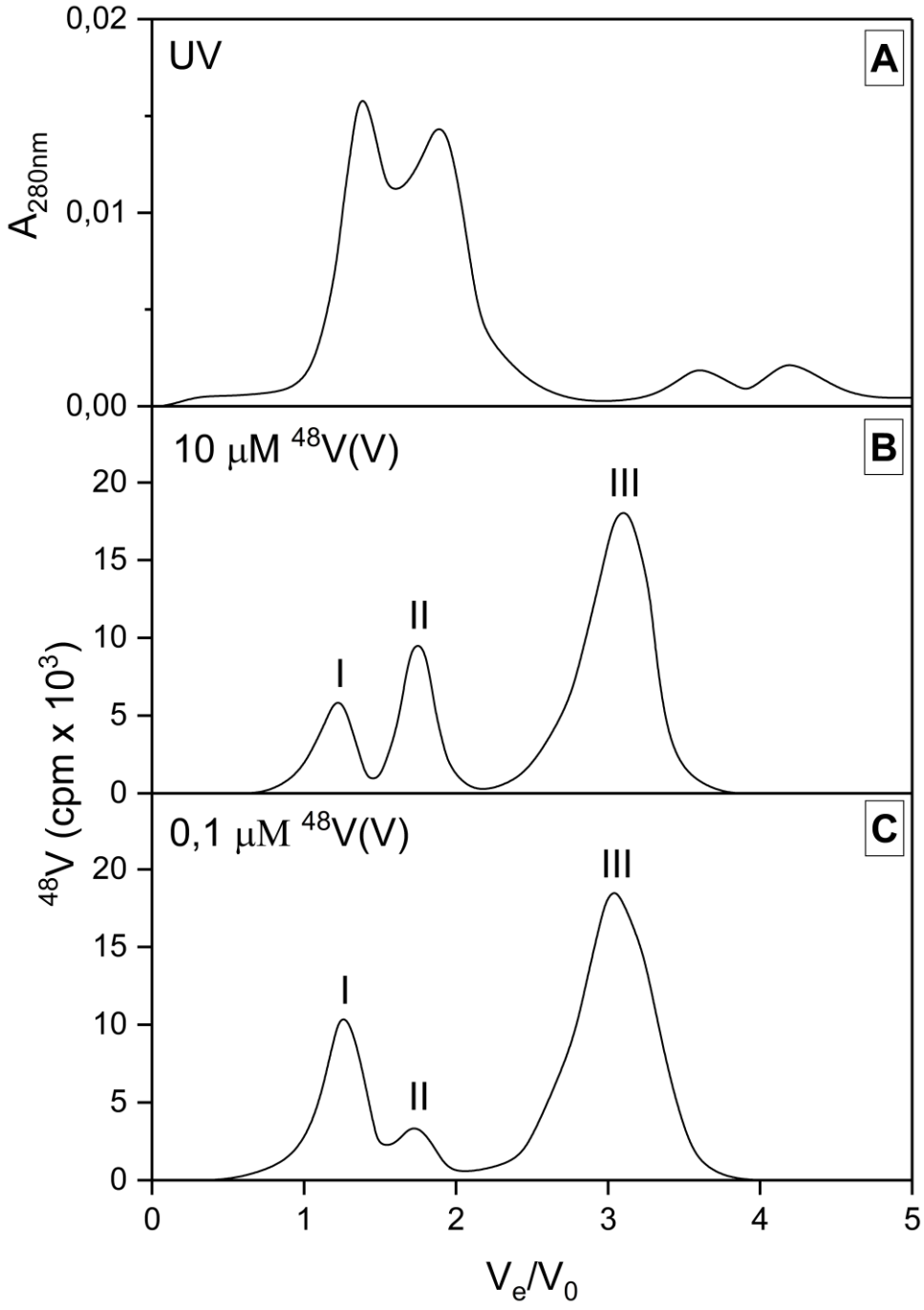

Fig. 8 ^{48}V elution patterns of Sephadex G-150 (2.5x110cm) of the Balb/3T3 48V-cytosol 72h after exposure to morphological transforming (10 μM, B) and non-morphological transforming (0.1 μM, C) concentrations of ^{48}V(V)

Exposure to 10μM of ^{48}V(V) (transforming dose) led to an altered intracellular partition compared to the exposure to 0.1 μM (non-transforming dose), being 20% of cellular ^{48}V shifted from cytosol to cellular organelles, particularly nuclei and mitochondria (Fig. 7). SEC of the ^{48}V-containing cytosols shows that the ^{48}V was eluted as three peaks: the first immediately after the void volume (^{48}V-peak I); the second in association with mol.wt of 70- 80 kDa (^{48}V-peakII). The third ^{48}V-peak III emerged in the position of the ^{48}V alone (Fig. 8). Interestingly, the relative proportion of the ^{48}V peak I and II changed at the transforming dose exposure (10μM) compared to that observed at non-transforming dose (0.1μM). Moreover, SEC of ^{48}V-cytosol after exposure to tetravalent vanadium shows also three ^{48}V-peaks which were quali- and quantitatively similar to those eluted after exposure to non transforming dose (0.1 μM) of pentavalent vanadium (chromatographic profile not shown). Determination of the oxidation state of vanadium of the three peaks by Chelex 100 chromatography (Table 5) shows that more than 96% of the ^{48}V was retained on the Chelex 100 resin, suggesting that vanadium was in the oxidation state +4. The only exception concerned the case of exposure to 10 μM of ^{48}V(V) for which the retention on the column decreased to 74.6% (oxidation state +4) while 25.4% was recovered in the eluate (oxidation state +5).

Table 5 Chelex 100 chromatography of ^{48}V containing peaks obtained by gel filtration of the ^{48}V-cytosol from Balb/3T3 cells exposed to 0.1 and 10 μM of ^{48}V(V)

	^{48}V (% of the total fraction)	
Dose (μM)	**Chelex 100 column**	**Eluate**
10 μM 48V(V)		
Peak I	97.3	2.7
Peak II	96.5	3.5
Peak III	74.6	25.4
10 mM 48V(V)		
Peak I	99.9	0.1
Peak II	98.6	1.4
Peak III	99.0	1.0

a: mean of 3 determinations, RSD<10%

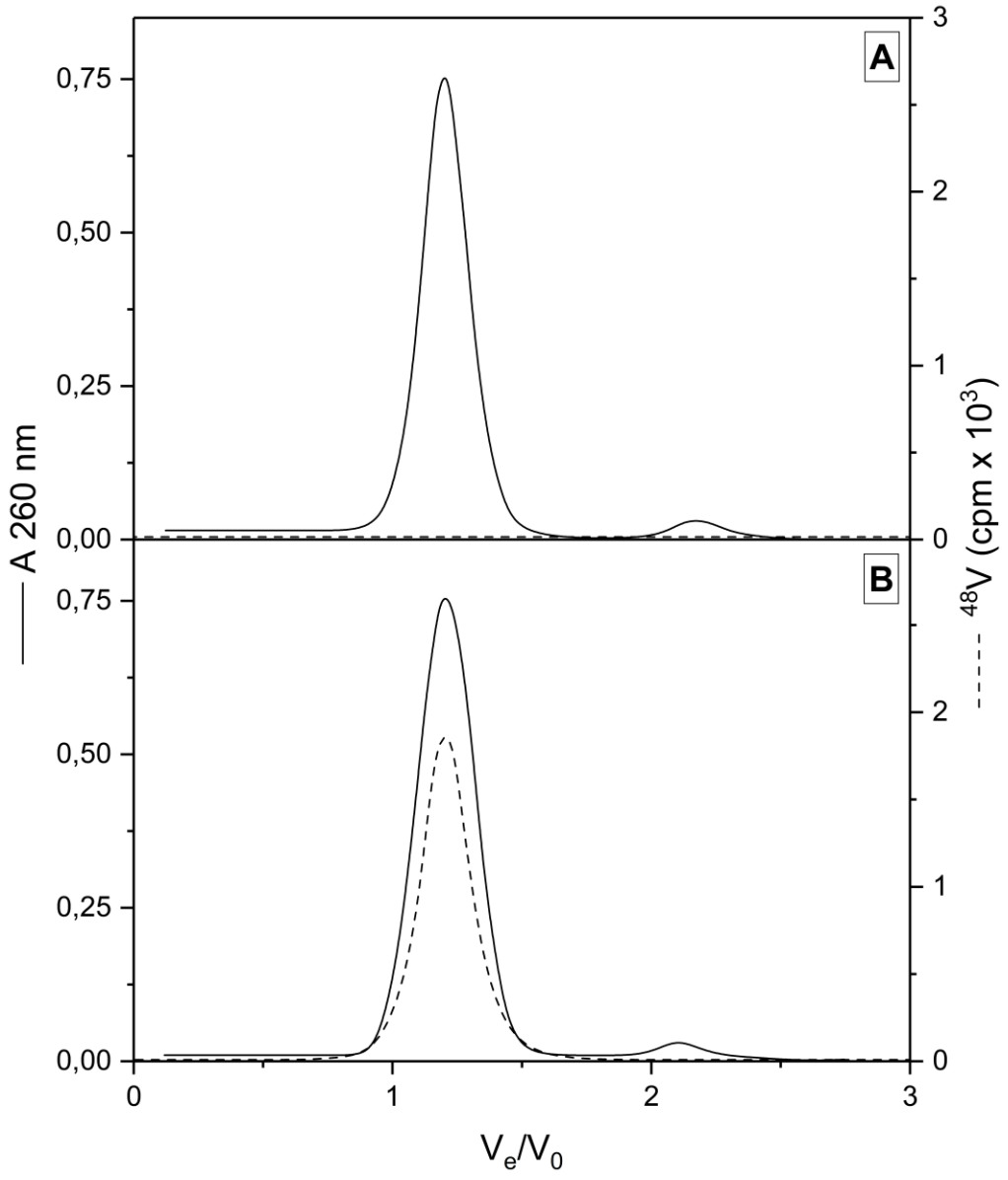

Fig. 9 The binding of 48V to DNA in Balb/3T3 cells.

A: 325µg of DNA was isolated from unexposed 5x10^{6}cells by NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) and incubated with 5µM ^{48}V(V). The incubation mixture was submitted to SEC on Sephadex G25 resin (1.6x40cm) previously equilibrated with 10mM Hepes, pH=7.2, recording UV and ^{48}V profiles in the eluate. **B**: 5x10^6 cells were exposed for 72h to 5 µM of $NaVO_3$ plus 5 µCi of ^{48}V in the same chemical form and highly pure DNA was isolated using a NucleoSpin Tissue kit. Then, the DNA fraction was chromatographed on Sephadex G25 resin as in **A**.

Size exclusion chromatography of a solution containing 5µM $^{48}V(V)$ and DNA previously isolated from unexposed Balb/3T3 cells led to the absence of ^{48}V in the eluate (Fig. 9A). On the contrary, SEC of DNA isolated from cells exposed to 5µM $^{48}V(V)$ shows an obvious binding of ^{48}V to DNA (Fig. 9B). This suggests that the formation of the ^{48}V-DNA adduct is cellular-mediated, probably previous bio-reduction of $^{48}V(V)$ to non-toxic and non-transforming V(IV) species.

In vitro studies on cells of human origin

Intracellular behavior of cadmium in $^{109}Cd(II)$ –exposed human umbilical cord blood (UCB) stem cells

Human haematopoietic stem cells (HHSC) are a well-characterized model for toxicological and carcinogenicity studies of environmental hazards including metal compounds [49]. They are multi-potent cells able to differentiate into several lineages, giving rise to more specialized cells of human body. We previously observed toxic effects in HHSC cells exposed to 10 µM of Cd (II) being 0.1µM considered a non- toxic doses [34] In this context, for a better understanding the mechanism of cadmium toxicity we have undertaken a study on uptake and intracellular fate of non –toxic and toxic doses of ^{109}Cd radiolabelled cadmium (II) in human haematopoietic CD34+ progenitor cells isolated from umbilical cord blood (UCB).

Table 6 Uptake of ^{109}Cd by UCB cells exposed for 72h to different concentrations of $^{109}CdCl_2$[a]

Dose (µM)	n° cells	pg tot	pg cell^{-1}
0.1	4.1×10^6	3.5×10^4	0.0085
1	3.3×10^6	2.5×10^4	0.076
10	1.4×10^6	9.8×10^4	0.70

a: mean of 3 determinations, RSD<20%

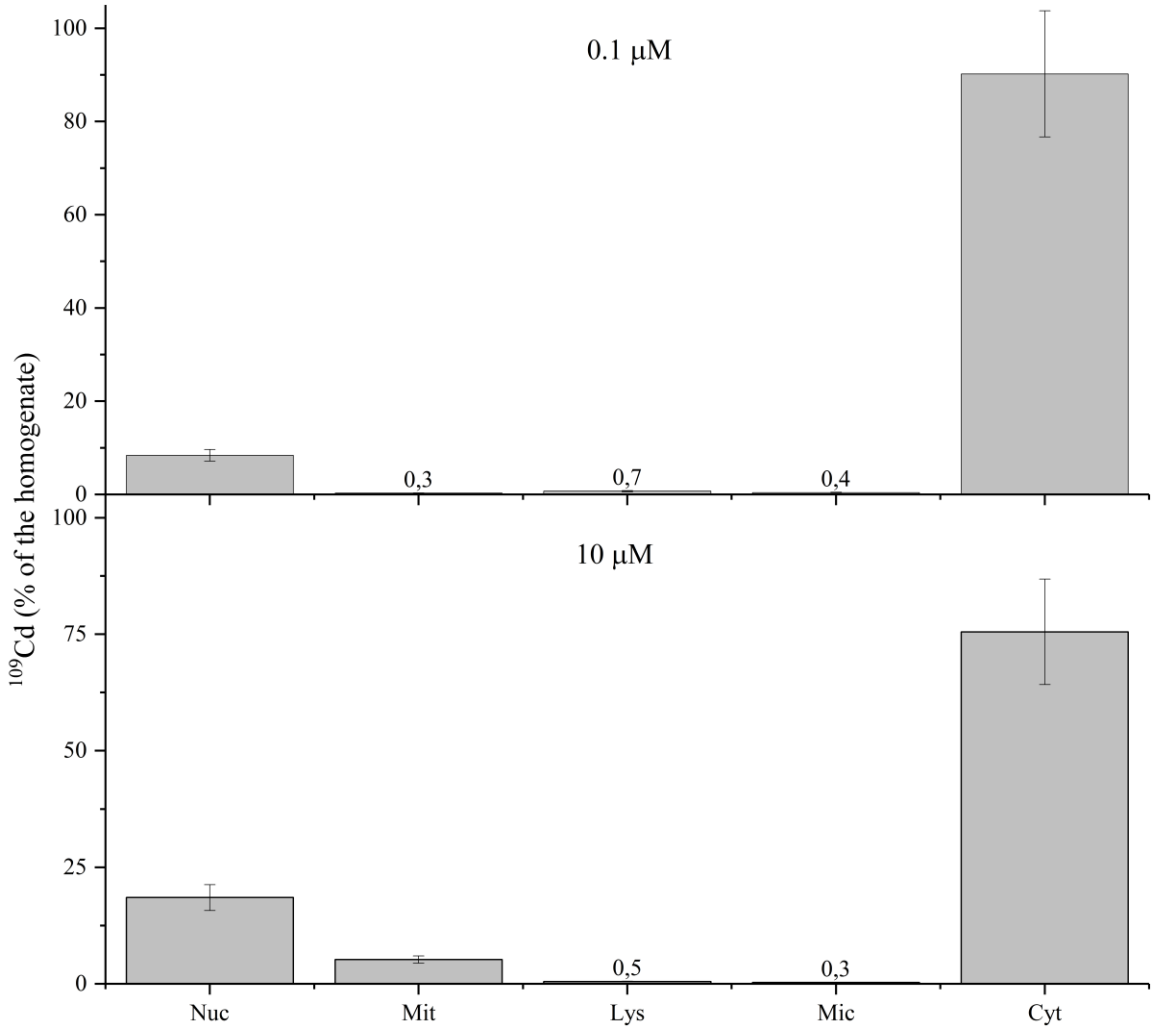

Fig. 10 Intracellular distribution of ^{109}Cd in UBC stem cells exposed to 0.1 and 10 μM of $^{109}CdCl_2$. The results, expressed as the percentage of the ^{109}Cd in the homogenate, are the mean of 3 experiments. Nuc(nuclear), Mit (mitochondrial), Lys (lysosomal); Mic (microsomal), Cyt (cytosol) fractions.

At 72h post-administration the uptake of ^{109}Cd was almost linear with its exposure concentration (Table 6). At 0.1μM 90% of the intracellular ^{109}Cd was recovered in the cytosol and the remaining almost in the nuclear fraction (Fig. 10) However, at toxic dose (10 μM) the ^{109}Cd in the cytosol declined to about 75%, the decreasing being matched by an obvious increasing in cell organelles such as nuclei and mitochondria. In any case, for

both exposure doses the elution profiles from SEC of the ^{109}Cd-cytosol shows the presence of a single ^{109}Cd peak in association with UV absorbing components with low molecular weight (about 10 kDa, most likely metallothionen) (Fig. 11). At toxic dose the presence of an "excess" of Cd in cellular organelles such as nuclei and mitochondria may be responsible of the cytotoxic effects induced by Cd in HHSC cells.

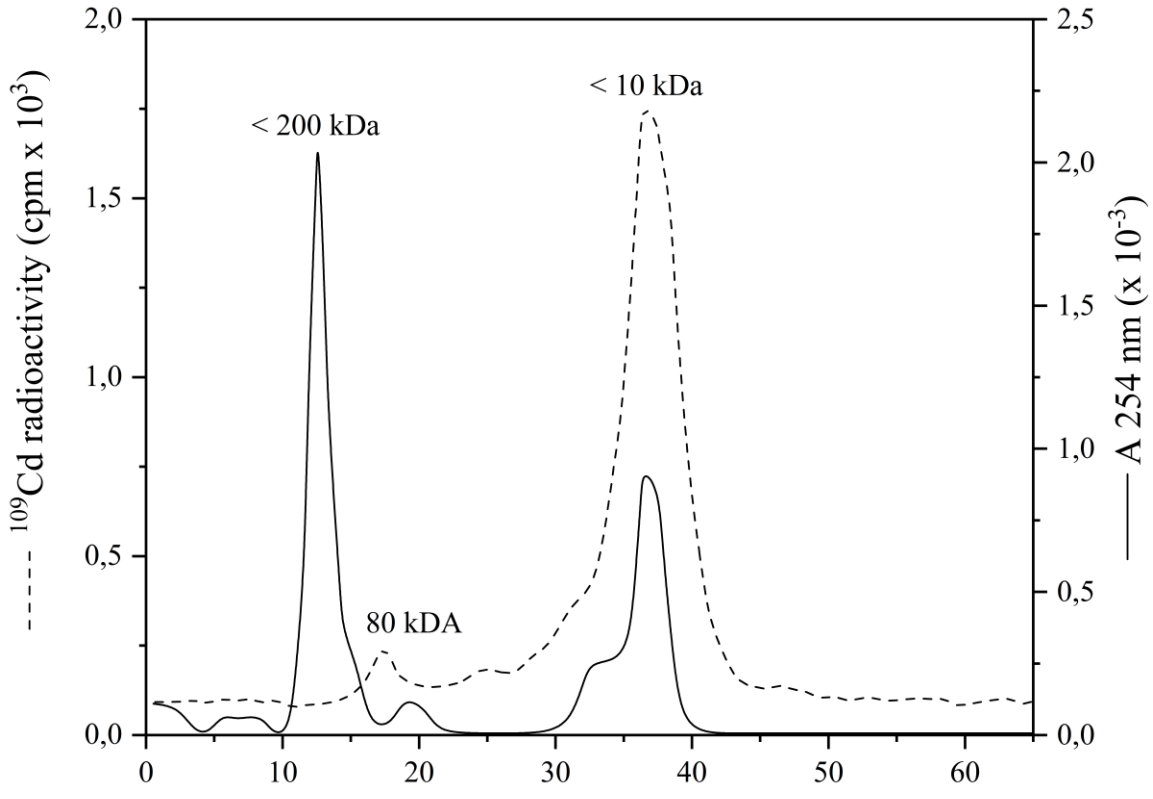

Fig. 11 Elution pattern of cadmium in ^{109}Cd-cytosol from human UBC stem cells exposed to 10µM ^{109}Cd as chloride. Eight hundredth µL of ^{109}Cd-cytosol were adsorbed on Sephacryl S200 column (1.6x30 cm, exclusion limit 2kDa) (Amersham Biosciences, Sweden) previously equilibrated with 44mM $NaHCO_3$, pH=7.4. Elution was carried out by the same buffer. 3mL fractions were collected and continuously monitored for the UV at 254nm using a Lambda 25 spectrophotometer equipped with a flow-through cell (Perkin Elmer, Italy). ^{109}Cd was measured in the same fractions. The Sephacryl S200 column was previously calibrated by using a kit of standard proteins of known molecular weight (Fluka, Milan, Italy)

Studies on humans

Trace metal reference values in general population

The assessment of health effects related to different trace element exposure conditions requires knowledge of "normal baseline data" (reference values, RVs) of trace elements in human body fluids and tissues. The RVs represent a fundamental prerequisite to identify anomalous trends of essential/toxic elements in general population as well as for clinical/toxicological assessment studies [20] In this context, we have undertaken a pilot study on the determination of trace metals in blood, urine and hair of inhabitants of different areas of the Sicily region. Table 7 reports the results concerning the analysis of Sb in blood, urine and hair. In the blood the levels of Sb were of the order of μg/L or less in agreement with recent evaluations on this [50]. Interestingly, differences can be indicatively observed. Inhabitants living in large cities (Messina, Palermo) or in highly industrialized areas (Milazzo) show the highest Sb concentrations, while the lower concentrations concern inhabitants of Pantelleria, an island in the south west of Sicily exclusively dedicated to agriculture.

Table 7 Antimony concentrations in blood, urine and hair of inhabitants of Sicily region, south Italy as determined by radiochemical separation NAA

		Sb content (Mean ± SD)		
Site	**n**	**Blood (μg/L)**	**Urine (μg/L)**	**Hair (ng g^{-1})**
Messina	53	1.66 ± 0.33	0.90 ± 0.41	214 ± 72
Novarasicilia	27	0.96 ± 0.21	0.55 ± 0.33	115 ± 91
Tortorici	28	0.91 ± 0.35	0.81 ± 0.35	141 ± 65
Milazzo	45	1.72 ± 0.44	1.55 ± 0.73	221± 110
Lentini	37	1.45 ± 0.63	0.67 ± 0.42	131± 96
Carlentini	31	1.56 ± 0.50	0.71 ± 0.26	146 ± 77
Palermo	56	2.49 ± 1.05	0.88 ± 0.40	212± 105
Pantelleria	48	0.41 ± 0.20	0.37 ± 0.23	75 ± 31

503 Cr overload in uremic patients under hemodialysis

504 It is now established that uremic patients on regular hemodialysis are at risk of deficiency
505 of essential trace elements and excesses of toxic trace elements, which can both affect
506 health [51]. Here we present investigations related to the chromium in patients under
507 regular hemodialysis. Two aspects are considered: an intradialytic Cr mass balance, and
508 the determination of the element in autopsy tissues of patients. During standard
509 bicarbonate dialysis the Cr concentration in outlet dialysis fluid significantly decreased
510 (Table 8) giving a mean net contribution to the patient of about 60 µg/dialytic session,
511 leading to a positive Cr mass balance. This is in agreement with the conclusions of a
512 systematic review on trace element status in blood of hemodialysis patients [52]
513 Moreover, the Cr imbalance established by our intradialytic mass balance study would
514 explain the obvious overload of Cr in autopsy tissues of patients under regular
515 hemodialysis (Table 9).

516

517 **Table 8** Intradialytic chromium mass balance during hemodialysis (4h standard
518 bicarbonate dialysis). Blood and dialysate samples were collected at the beginning and
519 end of dialysis together with the total (120 L) 4h outflow dialysate

	Cr content (µg L^{-1})		
	Mean± SD	Median	Range
Dialysis fluid:			
beginning dialysis	0.99 ± 0.61	0.72	0.49 – 1.85
end of dialysis	0.51 ± 0.12	0.52	0.37 – 0.65
Serum:			
before dialysis	1.14 ± 0.71	0.8	0.3 – 3.0
end of dialysis	2.56 ± 1.75	2.3	0.5 – 4.9

520

521

522 **Table 9** Cr concentrations in autopsy tissues of uremic patients under regular
523 haemodialysis (n=18) and control subjects (n=16) as determined by radiochemical
524 separation NAA

Tissue	Cr concentration (ng g^{-1} wet w.)					
	Patients			Controls		
	Mean ± SD	Median	Range	Mean ± SD	Median	Range

Brain	78 ± 47	62	20 – 81	3.8 ± 2.5	2.2	1.1 – 7.5
Cerebellum	117 ± 49	97	77 – 203	13 ± 11	9.5	2 – 32
Heart	501 ± 352	285	82 – 995	96 ± 40	101	30 – 120
Lung	704 ± 418	717	169 – 1204	105 ± 55	88	12 – 206
Kidney	376 ± 329	278	27 – 725	5.1 ± 4.4	4.7	2 – 16
Spleen	1117 ± 682	934	188 – 2015	32 ± 12	31	8 – 44
Liver	816 ± 389	534	43 – 1208	5.1 ± 2.2	3.9	1.9 – 8.5
Muscle	32 ± 15	30	8 – 58	2.7 ± 1.6	4.3	1.5 – 14.5
Skin	373 ± 335	199	61 – 1148	190 ± 109	145	33 – 350
Iliac crest	688 ± 591	480	165 – 1955	212 ± 180	165	70 – 540
Rib	981 ± 854	746	131 – 2906	296 ± 152	208	92 – 503
Toenails	1812 ± 822	1700	243 – 3380	1036 ± 587	897	102 – 1810
Pubic hair	101 ± 51	118	17 – 170	79 ± 44	79	21 – 158
Carpal tunnel	1591 ± 259	735	65 – 5980	–	–	–

Conclusions

The excellent sensitivity for a great number of elements, the high specificity, the multielement character, and the virtual absence of matrix effects, are the most outstanding advantages of NAA, which make very attractive for use in the ultra-trace metal analysis of tissues, cells and cellular components. The most decisive advantage, however, is its relative freedom from errors due to contamination of samples with endogenous material prior to the instrumental measurement. Thus, the characteristics of NAA make possible the simultaneous determination of trace metals at the sub-part-per-billion level in microsamples of biochemical materials, such as intact cells and intracellular components. In addition, NAA plays a fundamental role in the biomonitoring of trace metals in human tissues, covering the need of human information in as foreseen by the box “human data” of the parallelogram approach (Fig.1). The determination of Sb in biological specimens of normal population, and of Cr in the body of uremic patients under hemodialysis are typical examples in which high sensitivities are reached by radiochemical separations on neutron irradiated samples, in order to selectively isolate the induced radioisotopes.

The use of the radiotracers with high specific activity have proven to be of great value in studies at the intracellular and molecular levels on low doses of trace metals. Their characteristic radiation can be detected rapidly, simplicity and high sensitivity during cell fractionation and isolation of cellular components. The here given example of rats exposed to nanograms of radiolabeled $^{201+202}$Tl cover the need of information foreseen by the box "in vivo animal" of the parallelogram approach (Fig.1). This application shows how the use of $^{201+202}$Tl allowed the detection of pg levels of thallium, describing in vivo, for the first time, a covalent binding of the element with biochemical components of the testis, and establishing the long persistence of the element in different brain regions. We believe that such results, of particular interest in the environmental thallium toxicology, could not be achieved by other means, in vitro animal and human systems. ^{73}As, ^{109}Cd and ^{48}V were used in applications covering the need of information foreseen by the boxes "in vitro animal cells and human cells" of the parallelogram approach. Relevant findings have been achieved on the mode of action of these trace metals. In particular, the study by ^{73}As (III) in rat brain aggregates has shown that the inorganic arsenic is methylated even in this cellular model, being the degree of incorporation of ^{73}As cell-type dependent. The investigations with ^{48}V in a model of carcinogenic potential of chemicals (Balb/3T3 cells) has given, for the first time, evidence on the simultaneous presence of the two oxidation state +4 and +5 in the same cells exposed to ^{48}V(V), further reinforcing the thinking that the neoplastic transformation of Balb/3T3 morphology is dependent on the intracellular persistence of vanadium (V), and finally that the bioreduction of V(V) to V(IV) is a detoxication mechanism. The study by ^{109}Cd in human UBC stem cells suggests that at toxic dose the detoxification mechanism of cadmium (induction of metallothionein) would be saturated, leading to a redistribution of the element among cellular compartments, reaching levels in organelles, such as nuclei and mitochondria, capable of altering normal cell functions.

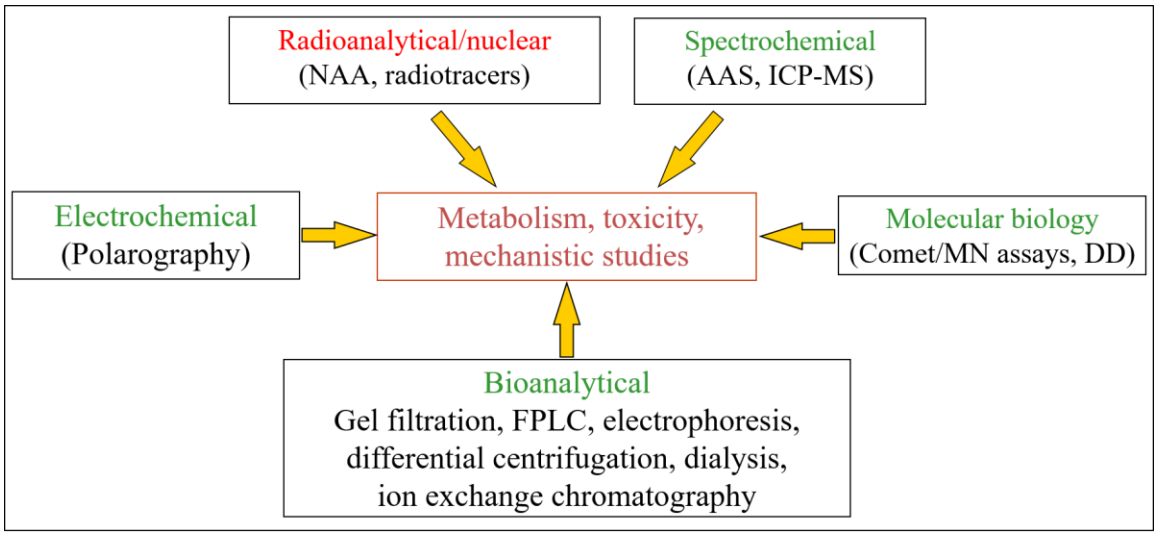

Fig. 11 Trace metal toxicology research: the integrated use of analytical techniques

In conclusion, the application of radioanalytical and nuclear techniques in combination with other analytical techniques (Figure 12) find an important place in trace metal toxicology research. In particular, investigations in cell culture is relatively new and, consequently, the potential of these techniques is far from fully exploited. However, the examples given here show that radiotracers give excellent performances in this field. The use of cell cultures is almost always directed toward investigations of the biochemical effects of trace metal exposure. The use of radioanalytical and nuclear techniques complement these investigations very well, adding information which is relevant in setting uptake-effect relationships, and in understanding the biochemical mechanisms of metal toxicity.

Acknowledgements

Acknowledgments of people, grants, funds, *etc.* should be placed here. The names of funding organizations should be written in full.

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