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Review

Nutritional and Methodological Perspectives of Zinc Ions and Complexes - Physiological and Pathological States

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Zinc, an essential element playing crucial roles in numerous physiological, but also pathological processes, is still one of the most studied elements. The main importance of this metal is especially based on the structural role of zinc ions in many proteins including the transcription factors, antioxidant enzymes, and metallothioneins. Due to the role of zinc in many crucial physiological processes, its homeostasis must be intensively controlled. The controlling mechanisms are based especially on the zinc transporters and low-molecular proteins metallothioneins (MT). Zinc deficiency has been related with different state of diseases. Here, we primarily aim our attention to zinc and nutrition reviewing the whole process from intake to determination of this metal. The function of zinc ions in proteins with particular emphasis to oxidative stress is discussed followed by intake mechanisms and the role of zinc ions in both physiological and pathological processes. Finally, we summarized the assays used for detection of zinc in complex biological matrices.

1. INTRODUCTION

Recent advances in understanding the human genome have been made possible due to intensive multidisciplinary cooperation involving the life sciences and technology. Genomics has succeeded in producing complete genome DNA sequences of different living organisms, but we are still some way from understanding the difference between the normal and pathological functions of cells and organisms. Currently, attention is directed towards proteomics providing information about protein localization, structure and function, and most importantly, interactions with other proteins. Recent improvements in high-throughput sample separation and mass spectrometry have impacted positively on the proteomic characterization of proteins in biological systems [1,2]. Metalloproteins are one of the most diverse classes of proteins, with the intrinsic metal atoms providing a catalytic, regulatory and structural role crucial to protein functioning [3].

Transition metals such as copper, iron and zinc play important roles in life as micronutrients, taking into account that their deficiency could result in disease [4-9]. In 1963 Prasad *et al*. penetrated the world of zinc in clinical practice with ill patients revealing for the first time the connection of zinc concentration to certain abnormalities in people from Iran and Egypt with different characteristic symptoms as dwarfism (due to slow growth), hypogonadism and delayed sexual maturation that disappear with zinc supplementation [10,11]. Zn is an essential trace element in many situations, in maintaining intestinal cells, bone growth, immune functions, being the most abundant transition metal in cells, playing a vital role in the functionalities of more than 300 enzymes, and in the stabilization of DNA and in gene expression [12,13]. It is distributed throughout the body, as it is part of many metabolites found in different organs and fluids. Approximately 60% of Zn in the body is localized in skeletal muscles, 25% in bones, 8% in skin and hair, 5% in liver, while only a small fraction (less than 0.1%) can be found in plasma [14]. In biological systems Zn is present exclusively in cationic form as Zn(II). Zinc ions are cofactors of more than 300 enzymes serving as electron acceptors or stabilizer of protein structure. In other proteins, Zn(II) is a component of specific domains, for example zinc fingers in transcription factors. At cellular level the concentration of free Zn(II) is strictly regulated, thus practically all Zn(II) is bound to either organic or inorganic ligands [15]. At the same time zinc ions circulate between the ligands resulting in changes in enzyme activities, protein-protein interactions and protein-nucleic acids interactions[16]. Except catalytic and structure functions of bound Zn(II), changes in free Zn(II) serve for intra- and intercellular signalization [17-19]. For this reason in this review Zn is used for all forms of Zn(II) present in human organism.

The Zn is mainly obtained through diet. Foods with higher content of Zn are almost always of the animal origin: especially oysters, pork, beef, veal, lamb, dairy products and some types of seafood. However, there are many more foods that serve as a source of nutritionally obtained Zn: nuts, seeds, pulses, whole grains, and with less with some fruits and vegetables [20]. All of these foods contain Zn, but usually it is associated with various proteins and nucleic acids that affecting its bioavailability. The Zn originating from plant foods exhibits a lower bioavailability due to the presence of phytic acid insoluble complexes poorly absorbable form.

Today, the state of zinc deficiency is prevalent worldwide [21] (the prevalence of Zn deficiency and childhood stunting is shown in Figs. 1A and 1B), but the problems caused by zinc deficiency in

societies are ignored. Because anomalies regarding zinc homeostasis generate a certain known symptoms, in many cases deficient patients are not detected. The aim of the review is to summarize the knowledge regarding chemical and functional aspects of zinc and biomarkers involved in human pathological and physiological statuses, and to discuss suitable methods for highly sensitive zinc determination in biological matrices.
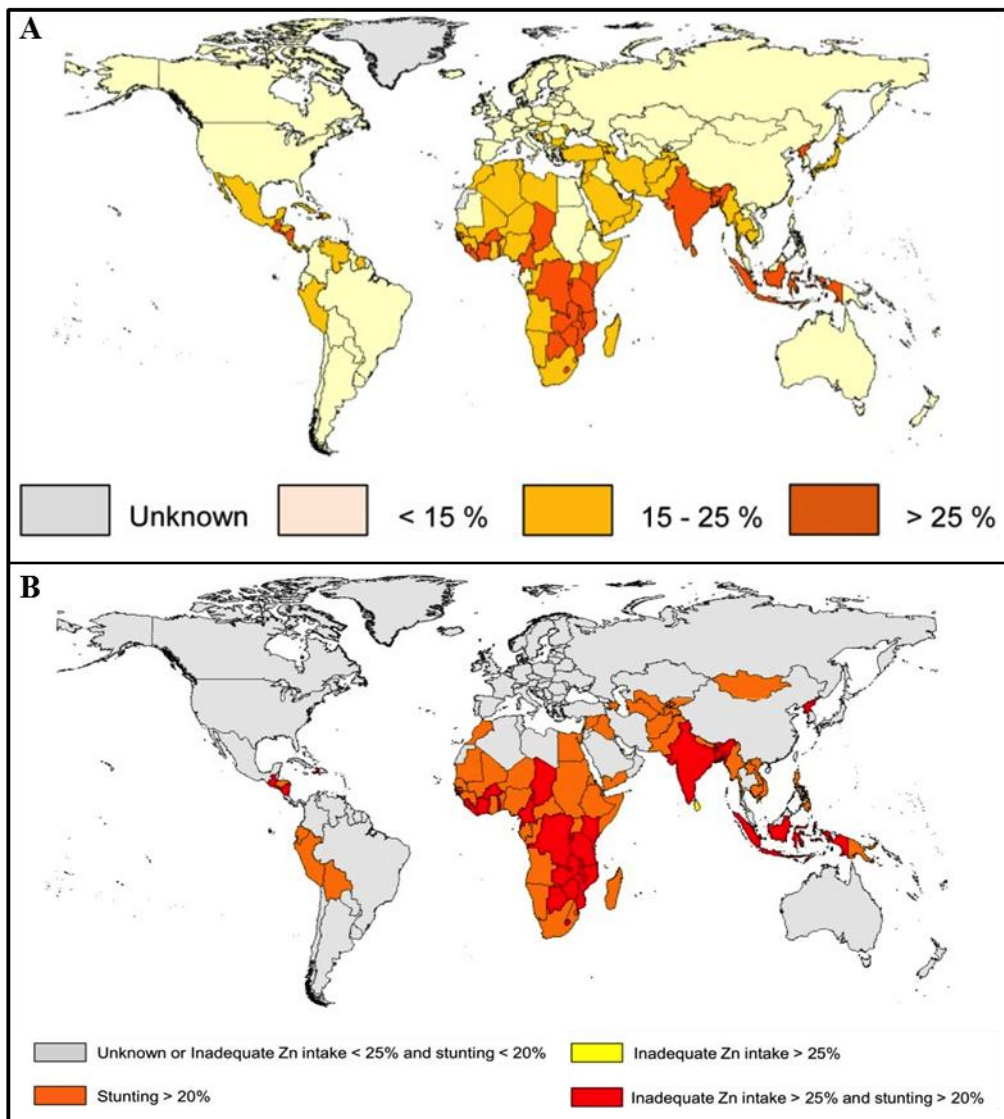
Figure 1. (**A**) Estimated country-specific prevalence of inadequate zinc intake, using the 2003-2007 time frame. (**B**) National risk of zinc deficiency based on the prevalence of childhood stunting and the estimated prevalence of inadequate zinc intake. Modified and adapted with permission from [21].

2. ZINC STATUS ASSESSMENT

In several chronic diseases, like atherosclerosis, ageing, age-related degenerative diseases, neurological alterations, specific malignancies, autoimmune diseases and Wilson's disease, zinc deficiency can complicate the clinical features, influence negatively immunological situation, decrease

antioxidant status, and imply generation of inflammatory cytokines. In these diseases, oxidative stress and chronic inflammation may play important etiologic functions [22].

Zn has a very important role in many enzymatic reactions: proteosynthesis, wound healing [23], DNA synthesis [24], cell division and immunity [25]. In addition, Zn also helps normal growth and development during pregnancy, child's growth and adolescence [26,27] and moreover it stimulates the senses of taste and hearing [28]. This pleiotropic activity requires detailed regulation, since it is necessary to introduce the Zn in the correct protein and at the right time. That is why we say that the bioavailability of Zn through diet and proper functioning of the entire protein system involved in their regulation is vital to maintain full health [29].

2.1. Associations with anthropometric parameters

Anthropometric measurements are necessary for a complete nutritional assessment, and regarding zinc status, these parameters are highly related to Zn biomarkers, mainly in infant population. Zinc can mediate growth through its influence on the synthesis and secretion of growth hormones and activity of insulin-like growth factors [30]. Zinc is involved in DNA and RNA synthesis which moderate critical metabolic pathways involved in growth such as cell transcription and replication; synthesis of collagen, osteocalcin, somatomedin-c, insulin and alkaline phosphatase; and differentiation of chondrocytes, osteoblasts, and fibroblasts [31,32]. Thus, the mediating effect of zinc on appetite may lead to growth impairment.

Zinc deficiency has been implicated in the pathogenesis of eating disorders, like anorexia nervosa [33]. In human populations, trials have shown that zinc therapy improved weight gain in anorexia [34-36]. Abbaspour *et al.* (2013) demonstrated a modest level of Zn deficiency (6-7%) in the mostly middle-class rural and suburban Iranian communities [37]. The relatively good zinc status in both populations was due to an adequate supply of dietary Zn from animal source foods, which provided almost half of the Zn intake and supplemented the bread and rice staples. The value of the Dietary Reference Intake (DRI) is established taking into account the balance of body Zn, by considering various aspects such as loss of endogenous Zn and bioavailability of Zn consumed.

In 1973, the World Health Organization proposed for the first time an amount of Zn recommended based on related Zn homeostasis studies [20]. DRI values may vary depending on the type of population. In the case of Zn there are differences in terms of age, sex and physiological condition of an individual set. Recommended intake of adults varies Zn, 8 mg/day for women, 11 mg/day for men [38]. Likewise, in growing children Zn nutritional requirements increase as it also increases their age. However, during gestation and lactation, the daily needs of women rise to 11-12 mg/day and 12-13 mg/day, respectively. In infants fed with milk that is not of maternal origin, Zn requirements increase with respect to those who consume breast milk because the bioavailability of ingested Zn is lower [39]. In either case, babies need higher amounts of Zn from 6 months of age, which is when it begins to decrease the amount of Zn present in breast milk due to yet unknown physiological factors.

2.2. Valuation of intake

Nowadays, we know that in the absorption process of Zn numerous transporters are involved. Most of Zn is absorbed in the small intestine by a saturable process that involves these transporters to ensure Zn homeostatic regulation at cellular level. Unlike what happens with other micronutrients such as iron, in the case of Zn absorption is directly dependent on consumption rather than their status [40]. When increasing the amount of Zn in the diet, the absorbed percentage decreases and *vice versa*. This capability allows for remaining regulated Zn absorption. Also, the gastrointestinal tract maintains Zn homeostasis adjusting endogenous losses to its absorption. Zn endogenous secretion has two components: an inevitable metabolic loss, and an extra loss which may contribute to Zn homeostasis increasing or decreasing its amount [41]. In fact, through various studies, Lee *et al*. (1993) and Sian *et al*. (1996) showed that when the amount of Zn consumed varies, the processes of absorption and secretion are adjusted until equilibrium [42,43].

However, when consumption is reduced below a threshold limit, the total amount of Zn absorbed may be insufficient. In these cases it is becoming apparent that problems may occur due to Zn deficiency in the body. This deficiency activates the release of Zn from tissue and clinical signs began to emerge. Although Zn is distributed throughout the body, not all tissues are mobilized with the same speed to help in keeping the Zn cell at homeostatic levels. Furthermore, the other part of the liver and bones are involved. Finally, skeletal muscles and other organs such as skin, thymus and heart are acting [44].

3. VALUATION OF BIOCHEMICAL PARAMETERS: ZINC BIOMARKERS

Zn deficiency is a state which can result from a variety of situations: insufficient food intake Zn, its poor absorption [45], or excessively high body weight losses (a through sweat and epithelial desquamation further) [46]. In either case, the effects on the body can be varied. However, no visible clinical symptoms usually occur except in those cases in which the lack of Zn is particularly severe. Discovering of accurate novel zinc biomarkers in healthy subjects is challenging enough. Lowe *et al*. (2009) carried out a systematic review from 46 papers to assess the usefulness of biomarkers of zinc status in humans and to determine which biomarkers appropriately reflect variations in zinc status in response to depletion or supplementation [47]. This study confirms that in healthy individuals, plasma, urinary, and hair zinc are reliable biomarkers of zinc status.

Regarding a human illness there are no biomarkers which are unaffected by the inflammatory responses associated with most infectious and non-communicable diseases, complicating the interpretation of zinc status biomarkers. Thirty-two potential methods of assessment of zinc status was reviewed in 2009 [47] and searching of new biomarkers for zinc status is still challenging, as shown in paper by Ryu *et al.* (2011), where combination of transcriptome, miRNA and cytokine analyses was applied to reveal potential new candidate protein and miRNA biomarkers of human dietary zinc depletion and homeostasis [48], or Grider *et al.* (2013) who analyzed whole plasma proteome in dependence on zinc depletion [49]. In addition to zinc concentration, levels and activities of zinc

proteins indicate to zinc status and can provide additional information about zinc availability in the organism [50]. These proteins are involved either in zinc storage such as albumin [51], metallothionein (MT) [52,53], zinc transport [54,55], regulation of transcription or immunity system [56]. Another group are zinc-dependent enzymes, such as serum alkaline phosphatase [51], lactic dehydrogenase, purine 5′-nucleotidase [57] or SOD [58]. Zinc status can also be assessed indirectly for example by determination of DNA integrity [59,60] or antioxidant status.

3.1. Zinc in health

Adequate zinc nutrition is necessary for normal growth, development, protection from infection, and pregnancy. The total body zinc can be divided into rapid and slow exchangeable pools. The slow pool represents 90 % of whole-body zinc and its components are skeletal muscles and bones. The rapid pool is composed of the most metabolically active zinc forms; it includes zinc from plasma and extracellular fluid and in liver, kidney, pancreatic and intestinal tissues [61,62]. Serum or plasma zinc concentrations, which are the most frequently used for assessing zinc status, vary with age and sex. For both males and females, serum zinc levels were lower in childhood, with maximum level during adolescence and decreasing slightly with age. From adolescence to adulthood, men had higher serum zinc concentrations with greatest differences for 20 - 40 year age group [63].

The influence of genetic factors on individual's serum zinc concentration is unknown. 104 genes have been identified that responded positively to zinc supplementation and 86 genes, which responded negatively. These genes were involved in zinc transport, trafficking, and homeostating [64]. Because of homeostatic mechanisms, the serum zinc concentration is maintained within a narrow range, even when a broad range of zinc doses above and below theoretical requirements are consumed. Thus decreases of serum zinc level occur after severe or prolonged zinc depletion and increase rapidly after its repletion [65]. There is evidence for effective homeostatic mechanisms allowing human adaptation to low zinc intake and that zinc deficiency may occur even in circumstances of apparently adequate intake of this micronutrient. This can be explained by two factors: variation in the bioavailability of dietary zinc and the diminution in one or more metabolically important body pools of this micronutrient. Compatible with this possibility are the observed correlations between estimates of the quantity of zinc that exchanges with zinc in plasma within 2 days and both the dietary zinc intake and the quantity of zinc absorbed [66].

The population most affected by such deficiency mainly includes pregnant and lactating women, newborns whose only food is breast milk, children with poor food consumption of Zn, and the elderly. Despite this, if we move to places with lower economic and food resources, Third World countries, then the problems due to a lack of Zn are often standardized to the entire population [45]. This is due to a widespread malnutrition between almost all of the inhabitants of these countries. Zn deficiency in newborns is usually associated with transit occurs when the child begins to consume external food. Zn content in human milk undergoes changes with time so that, at the time of delivery generally has relatively high concentrations (> 3 mg/L) and these will abruptly decrease to very low concentrations (<1 mg/L) after 6 months. The mechanism by which this reduction of Zn occurs in

breast milk is not yet known occurs, however this physiological pattern is well documented and not dependent on the consumption of dietary Zn in the mother [67]. In fact, it is consistent with the age at which it is assumed that the baby should begin to consume foods and consequently acquire Zn by external sources. It is at this moment particularly important likelihood in children suffering from a deficiency of Zn, It is particularly important that children suffering from a deficiency of Zn, follow a proper diet, rich in foods with low Zn and phytic acid etc. [68].

Also, in elderly, where Zn deficiency may aggravate any condition or disorder from which they are suffering, while increasing the risk of degenerative diseases associated with ageing. In these cases, Zn deficiency is often associated with consuming less than the recommended daily intake Zn. Stewart-Knox *et al.* (2005) stated that this poor consumption of Zn occurs in response to lower energy requirements with the elderly and sensory impairment [69]. But there are other theories: genetic alteration of some transporters is also considered because of the decreased absorption of Zn in the elderly [70]. In either case, the lack of Zn in the bodies of the elderly, is the cause of the availability of Zn ions to intracellular level [71] and as a result it is reduced and is responsible for the general situations of risk to the health of these people.

3.1.1. Modulating factors in health

Several factors have been described as potential modulators of micronutrients needs. In the case of zinc, it is known that *physical* activity improve the daily recommendations for this element, as its deficiency could trigger changes in the antioxidant, musculoskeletal, immune and inflammatory systems, resulting in declining in performance and fracture risk, among others. Physical activity carried out on a continuous basis and at certain intensity derives in an imbalanced nutritional status. As described previously, adequate zinc is needed for the integration of many physiologic systems such as immunity, reproduction, taste, wound healing, skeletal development, behaviour, and gastrointestinal function. This array of zinc dependent functions suggests that zinc status should regulate work performance [72].

Active subjects generally consume less zinc than recommended, 8 and 11 mg/d for women and men, respectively, with special emphasize in sport athletes who restrict food intake [73]. Attention to zinc status as a potential factor affecting physical performance, serum zinc has been shown to be essential. However, there is limited data available on the relationship between performance and zinc status, but physical activity seems to correlate positively with blood zinc level. The outcomes of the studies about changes in the concentrations of zinc in blood in physical effort situations have been determined by changes of zinc in serum and urine in response to aerobic endurance and muscular strength [74]. Acute and chronic effects of vigorous physical activity, on distribution of elements in young amateur boxers were related to the decrease on zinc serum levels [75]. Low circulating zinc concentrations in different tissues have been reported in competitive swimmers[76], and runners [77] and appear to be modulated by exercise. Several authors confirmed [78] the lower intensity and character of the physical exercise with the increased the body requirement for zinc. Dietary zinc may

have been inadequate in the athletes with the decreased circulating zinc. These findings provide the first evidence of impaired metabolic response during work when dietary zinc is suboptimal [73].

Zinc generally maintains an activity with enzymes that neutralize free radicals effects resulting from the oxidation stress caused by exercise. It has been reported that dietary deficiency of zinc may be associated with decreased tissue levels of SOD, possible peroxidative cell damage, and increased ROS generation [79] and the increase hepatocytes sensitivity to the free radicals-induced damage [80]. Recently, Zhao and coworkers (2015) found that plasma zinc was positively correlated with Cu/Zn SOD [81]. This result suggested that zinc is an antioxidant agent and decreases ROS, required for Cu/Zn SOD activity in male basketball athletes. It is also assumed that MT plays an important regulatory role and the high affinity to hydroxyl and superoxidative radicals demonstrating that the exercise a weaker anti-oxidative defence and MT taking up the function of an antioxidant [78]. The maintenance of the redox balance may partly result from the integrative effect of augmentation of individual antioxidant in adaptation to chronic exercise. Such adaptive endogenous processes in athletes might be associated with their habitual intakes of antioxidant nutrients [80].

There is limited data to imply that zinc status may affect physical performance. Preliminary observations suggest that changes of zinc in serum and urine are related to the type of exercise performed, which are higher when there is a big impact on muscular tissues [74]. Because these muscle functions rely on recruitment of fast-twitch glycolytic muscle fibres, it may be hypothesized that zinc supplementation enhanced the activity of lactate dehydrogenase, a zinc-dependent enzyme. Interpretation of the significance of these findings is confounded by the high level of zinc supplementation, which suggests that any biological effect on performance was a pharmacologic response. Ability of the body to use the administered zinc and limited duration of the zinc supplementation raise other concerns [73]. Also of interest is that zinc status in athletes appear to be related to immune cell number and function in comparison with non-athletes both before and during the four weeks of intensified training in runners in spite of plasma zinc concentration and immune markers remaining constant during the study [77]. Requirements for zinc are generally higher in athletes than in normal population because of increased losses in sweat and urine. However, excesses of some minerals (particularly zinc) can impair immune function and increase susceptibility to infection [82].

In summary, evidences that physical exercise suggest that exercise mobilizes zinc homeostasis related with the growing interest in micronutrient deficiencies, and athletes are at risk of zinc deficiency if they have an unbalanced diet. Further high-quality studies using these or new biomarkers are required for which there is limited data in physical exercise.

3.2. Zinc in disease

Today Zn homeostasis and especially its deficiency have been involved in numerous diseases from very different backgrounds. Neurodegenerative diseases such as Parkinson's or Alzheimer's disease (AD), liver failure, renal dysfunction, heart problems and different types of cancer are some of the diseases that have been linked to abnormalities in the homeostasis of Zn. Thus, this issue has

become in recent years the object of study of many researchers who want to know the mechanism by which these diseases develop.

However, as mentioned above, the lack of this mineral in the body may be due to various factors such as the lack of absorption of Zn, either as a result of any condition which enhances the appearance of such shortcomings as due to genetic problems that prevent proper absorption of Zn intestine. There is evidence that in hospitalized patients there is a high prevalence of malnutrition, due to reduced food consumption or increased nutrient requirements are increased. WHO and UNICEF recommend 20 mg zinc supplements for 10-14 days [12,83]. Zinc recommendation depends on nutrition type; those that are almost the same as healthy people on those with enteral nutrition. In critically ill patients they usually have artificial nutrition enteral or parenteral. For parenteral nutrition zinc can be calculated using a factorial method and has been established as an average of 2.2 mg/day [84]. In a randomized study in critical patients it has been shown that if they receive high intravenous of zinc, selenium and copper over 8 days, compared with controls of a normal dose, in patients with a high dose, the infection and income time in ICU is low [85]. Another study in septic mice show zinc deficiency leads to an excessive activation of NF-κB [86]. In paediatric children it has been observed that low plasmatic zinc levels are associated with non-survivors of septic shock and many genes are under regulated [87].

The extreme symptoms of zinc deficiency are characteristic for *acrodermatitis enteropathica*, as a consequence of mutation of the SLC39A4 gene, which affects the synthesis of ZIP4 zinc transported resulting in intestinal malabsorption of zinc. The clinical picture of severe zinc deficiency includes growth and development retardation, skin lesions, diarrhoea, hair loss or even alopecia, decreased sense of taste and smell together with anorexia, increased susceptibility to infections, behavioural and cognitive disorders and impaired vision [88].

Zinc is important for the immune system, activating T cells, thus it generally has anti-inflammatory and antioxidant properties [89,90]. It has been described as a protective role in many diseases for example viral infections like the common cold [90] or a HIV infection. Zinc has an effect in patients who especially suffer from zinc deficiency [91]. It also has been shown that zinc has an effect in infections such as hepatitis C, decreasing any gastrointestinal disturbance, body weight loss, and mild anaemia. Also, zinc has protective effects in bacterial diseases, such as diarrhoea, leprosy, tuberculosis or ulcer caused by *Helicobacter pylori* [91]. In asthmatic patients zinc deficiency the symptoms of the disease are increased by 4 to 5 times; zinc plays an important role in diseases the airways. In a meta-analysis it was demonstrated that serum zinc has a beneficial influence in osteoporosis [92]. In alcoholic patients it has been documented that there is a zinc deficiency in liver and this can lead to cellular death [93]. It has been found in cirrhotic patients that zinc deficiency influences disease severity, infection and poor transplant survival [94]. Zhou and coworkers exposed administered murine experimental model with decreased liver concentrations of metallothionein, first to alcohol and then afterwards to zinc. It was shown that alcohol induces degenerative morphological changes and subsequent oxidative damage is increased. In mice where zinc was administered after alcohol, zinc damped the toxic effects of alcohol [95].

Scientific evidences have shown an important role of zinc and recently many researchers have studied the effect of zinc deficiency in different illnesses and how zinc supplement can improve certain

diseases. Hopefully we can find these magical properties of zinc and together with other nutrients we can maintain our health better. Among the diseases in which zinc is mainly involved, neurodegenerative pathologies as AD has been recognized as a health worldwide problem affecting elderly people and increasing continuously [96].

3.2.1. Zinc, neurodegeneration and aging

The process of ageing has been explained on the basis of two different theories, namely "programmed" and "damage" theories, which likely inter-cross their effects in a complex way [97]. Therefore, ageing neurodegeneration might be considered as a consequence of an multifactorial cellular process [98] in which oxidative stress is involved as one among others factors together with the deregulation of metal homeostasis in neuronal cells (e.g. zinc [99]). From the seminal Harman's work [100], the oxidative cellular damage produced by an uncontrolled production of free radicals has been recognized as a primary pathway which provokes the loss of neuronal capacity in old age. From this point of view, it results plausible to link oxidative stress in aging with neuro-pathologies characterized by neuronal damages associated with oxidative processes and a decrease in antioxidant defences.

AD is characterized by synaptic loss and also by apoptotic neuronal deterioration which have been attributed to the appearance of (i) beta-amyloid proteins (Aβ), a sort of intrinsically disordered proteins [101], which can clump together to form extracellular amyloid plaque deposits, and (ii) intraneuronal neurofibrillary tangles composed of the protein tau in a hyperphosphorylated form [102]. Great effort has been dedicated to the amelioration of the symptoms of this neurodegenerative disease, based mainly on a clinical approach so-called "amyloid cascade" process which hypothesizes the presence of Aβ fibrils and plaques at the start of a number of steps which lead to the appearance of neurofibrillary tangles accompanied by neurodegeneration and clinical signs of dementia [103-107].

During the last two decades this hypothesis has provoked great attention and also an interesting debate since some findings seem not to fulfil the predictions coming from the "amyloid cascade" hypothesis, especially those pointing to a possible treatment of AD by reducing the Aβ amyloid plaques [108-111]. In addition, some positive outcomes in clinical trials related to effects on tangle formation [112] do not appear to be consistent with several findings [113-116] which identify Aβ smaller aggregates ("soluble oligomers") as the main toxic form of Aβ rather than Aβ fibrils. However, a recent study on Aβ fibrils, highlights how these aggregates present in senile plaques, retain redox activity while they remain bound to the Cu(II) ions, giving rise to ROS generation from H_2O_2 degradation [117]. Zn(II) interferes with H_2O_2 degradation if it is coincubated with Aβ fibrils and Cu(II) probably because of the redox-inert features of Zn(II) which elicits: a) a competitive displacement of Cu(II) from their binding sites or a Cu(II) relocation in a low-affinity secondary binding site [118,119], or b) a disruption of the Aβ aggregate morphology which contributes to reduce the redox ability of Cu(II)-Aβ complex [117]. These conclusions coincide to some extent with the high dynamics exhibited by labile metal-Aβ associations examined by Faller et al. [120]. Besides which,

other studies have been focused on metal dyshomeostasis in the brain ("metal hypothesis") and its possible involvement in the pathogenesis of AD and other related degenerative diseases [121-126].

In this case, AD etiology is considered to be linked to an abnormal accumulation in brain of metal such as iron, copper and zinc [127,128] with a capacity to form insoluble complexes with Aβ molecules. These complexes are characterized by an increased resistance to metalloprotease-2 [129] and the ability to originate ROS [130,131]. However, it is important to note that still remains unclear whether metal deregulation could be a cause or consequence of the presence of Aβ aggregates or the result of different events coming from AD etiology. In this scenario Zn can play an important double role characterized by a pro-oxidant or antioxidant activity [122]. In a number of reports a Zn excess correlates with the formation of toxic amyloid plaques [125,132-135] involved in the direct production of oxidant species. This correlation is established not only in synaptic space but also in intraneuronal location in which an increased cytosolic levels of Zn in neurons containing neurofibrillary tangles [136] or the mobilization of intracellular Zn, have been related to the oxidative stress in neurons [137]. This point has recently been reviewed by McCord and Aizenman [138]. Metal-Aβ peptides association under physiological conditions is seen facilitated by the amount of Zn released in some synaptic sites. It has been indicated that this kind of labile association metal-amyloid peptides are increased in AD [139]. In principle, the situation is completely different in the cytosolic medium, Cu and Zn affinity for metalloproteins is high enough to leave "free" Cu(I) and Zn(II) intracellular concentrations in the femtomolar to attomolar range and nanomolar to picomolar range, respectively [140,141] what it means that Aβ, with an affinity much lower than metalloproteins, should find environments with an increased amount of "free" Zn to get a binding with the metal.

Intraneuronal zinc homeostasis is mainly kept through binding to metallothionein, particularly MT-3 the primary isoform present in neurons [142,143]. Nevertheless, the close control of metals such as Cu and Zn can be modified by mild oxidation conditions. Thus, MT-3 oxidation under these circumstances produces Zn release[144] which may lead to a number of detrimental events [145,146] following the increased cellular Zn levels. In this case, it is possible that the mitochondria, which apparently co-regulates intracellular level of Zn along with MT-3, may take the Zn in excess released in MT-3 oxidation [147,148] provoking in this way interferences in electron transport chain generating ROS [149-152].

4. CHEMICAL AND FUNCTIONAL ASPECTS OF ZINC

Zinc, the 23rd most abundant element in the earth's crust, having atomic number 30 and atomic weight 65.37, is vital in the living world. Pure zinc is a bluish-white, shiny metal, and is amphoteric in nature. Zinc, being colourless and diamagnetic, is invisible to most spectroscopic methods [153]. At first sight, these non-interesting chemical facts on the zinc are, on the other hand, closely related to the fact that zinc can really be considered as The Metal of Life as Kaur and colleagues entitled their recent review [154].

Most of human body zinc(II), approx. 98%, is localized in the intracellular compartment [155,156]. Its total intracellular concentration is in a range within hundreds of micromoles, thus it is

approx. 10-fold higher compared to serum levels [141,157-159]. Most of the intracellular Zn(II) is bound to or at least associated with proteins and peptides [160]. Thereof, app. 90 % is tightly bound and the rest (10%) is bound with relatively low affinities, forming a reactive Zn(II) pool able to interact with other intracellular substances [161]. The last, very small fraction (approx. < 0.01‰ of total cellular Zn(II), ranging from pM to single digit nM includes free Zn(II) ions [141]. The tightly bound Zn(II) ions occur mainly in metalloproteins within metalloenzymes and nucleoproteins and acts as the structural components of these biomolecules or as an enzyme cofactor. This fraction can be considered as an immobile nonreactive Zn(II) pool [141,162]. The rest of the Zn(II) ions fraction, which acts as a mobile reactive form [163,164], is bound to low molecular weight compounds (amino acids as cysteine, histidine, proline), protein metallothionein (MT) or organic acids (citrate, oxalate) [165-167]. If there is a focus on cellular functions of Zn(II), determination of total cellular Zn(II) concentration is not of such importance as the determination of mobile reactive Zn(II) [161].

Zn(II) as the only transition metal lacking redox activity is an essential part of app. 10% of human proteins [162,168]. Due to a great portion of the zinc-depending proteins it is not surprising that Zn(II) is involved (through these proteins) in numerous key intra- and extracellular processes including proliferation, differentiation and apoptosis [9,169-171]. Zinc-dependent enzymes can be found in all classes of enzymes, i.e. oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases [141,172]. These enzymes include so-called zinc finger domains, repetitive sequences with ability to bind Zn(II) ions. Those domains consist of mostly histidine and/or cysteine. Zinc fingers are able to form complex with DNA based on interactions between α-helix of the zinc finger and DNA specific bases [173]. Function of zinc fingers consists not only in DNA recognition and transcriptional activation, but also in RNA packaging, protein folding and apoptosis, in which regulation is important not only in development of tissues, but also in neoplastic transformation and proliferation [169,170].

Based on the involvement of Zn(II) in the complex regulatory network, precise mechanisms to maintain intracellular Zn(II) level exist [174]. Zn(II) pool is maintained by two types of proteins: (a) zinc-binding proteins (mostly by metallothionein), which act as buffer and donor of Zn(II) for intracellular processes, and (b) zinc transporters, which are responsible for zinc fluxes into/from cells and organelles. A key regulator of intracellular free zinc level is metal regulatory transcription factor 1 (MTF-1, also called MRE-binding factor) [175]. This 753 amino acids transcription factor directly responds to the elevated Zn(II) level and induces the transcription of MT and main zinc transporter responsible for its export, ZnT-1 (discussed below) [176-178]. This auto-regulatory loop maintains narrow optimal limits of intracellular Zn(II): when the level of MT and ZnT-1 is elevated, more free Zn(II) may be buffered (i.e. bound to MT) and more zinc may leave cells (through larger amount of membrane transporters) [7].

4.1. Zinc transporters

The sophisticated control of systemic and cellular zinc homeostasis is essential for human health. Therefore, a number of proteins such as various zinc permeable and transport membrane proteins and MT are employed in the body for this control. Zn(II) cannot freely pass through

membranes, thus, special membrane transporters have been developed in a cell. Zn(II) transporters are transmembrane proteins, which transfer Zn(II) ions through cellular membranes [7,174]. Most of them are localized both on plasmatic and on organelle's membranes.

Two families of mammalian zinc transporters exist. Zinc-Iron Permease transporter (ZIP) also called Zrt-Irt-like protein, or solute-linked carrier 39 (SLC39) family and Zinc transporter (ZnT, SLC30) family [179,180]. ZIP transporter family is responsible for the influx of Zn(II) ions to the cytoplasm, in other words for the transporting of Zn(II) ions from extracellular compartments or from intracellular organelles to the cytoplasm. ZnTs are responsible for the opposite action, i.e. the efflux of Zn(II) ions from cytoplasm (transport from cytoplasm to the organelles or to the extracellular matrix) [181]. Transport mechanisms of these proteins are not fully understood, however, it is expected that there is ATP-independent facilitated diffusion, secondary active transport or symport mechanism [174].

Regulation of expression of both transporter families is not yet fully clear, however, in general, Zn(II) has antagonistic effect on transporters: whereas low Zn(II) load induces expression of zinc importers - ZIPs, high Zn(II) level induces expression of zinc exporters - ZnTs [181]. However, accumulating evidence has revealed that the coordinated zinc mobilization by ZIP and ZnT transporters is indispensable for maintaining zinc homeostasis. Thus, it plays critical physiological functions and profoundly affects health positively or negatively, as it is involved in a wide variety of diseases. Relative to the handling of dietary zinc is the involvement of ZnT1, ZIP4, and ZIP5 in intestinal zinc transport, involvement of ZIP10 and ZnT1 in renal zinc reabsorption, and the roles of ZIP5, ZnT2, and ZnT1 in pancreatic release of endogenous zinc. These events are major factors in regulation of zinc homeostasis. Other salient findings are the involvement of ZnT2 in lactation, ZIP14 in the hypozincemia of inflammation, ZIP6, ZIP7, and ZIP10 in metastatic breast cancer, and ZnT8 in insulin processing and as an autoantigen in diabetes [180].

4.2. Metallothionein

MT (Fig. 2) is currently classified in 15 families [182]. Mammalian MTs are single-chain polypeptides of 61 to 68 amino acid residues. Position and number of the cysteine residues are highly conserved and forms cys-x-cys, cys-x-y-cys, and cys-cys motifs, where x and y are non-cysteine amino acids. There are no free thiol moieties, and divalent metals are bound by sulphur atoms in thiolate clusters with a tetrahedral geometry [163]. The binding affinity varies between metals with Cu having the greatest stability constant followed by Cd and Zn. 18 different metals may bind to MT, but only Cu(I), Cd(II), Pb(II), Ag(I), Hg(II) and Bi(II) can displace Zn [183,184]. MT has two domains: the more stable α domain (C- terminal), which incorporates up to four divalent metal atoms, and the more reactive β domain (N- terminal), which contains only up to three. The exchangeability depends upon the metal species, and under *in vivo* conditions, MTs exist mainly in Zn form or as mixed-metal proteins. The tertiary structure of MT is dynamic and Zn and Cd exchange rapidly within the β domain, more slowly in α domain, and may also be exchanged with other ions bound to intracellular ligands. MT has also been found to donate metal ions to higher-affinity ligands or other proteins [185].
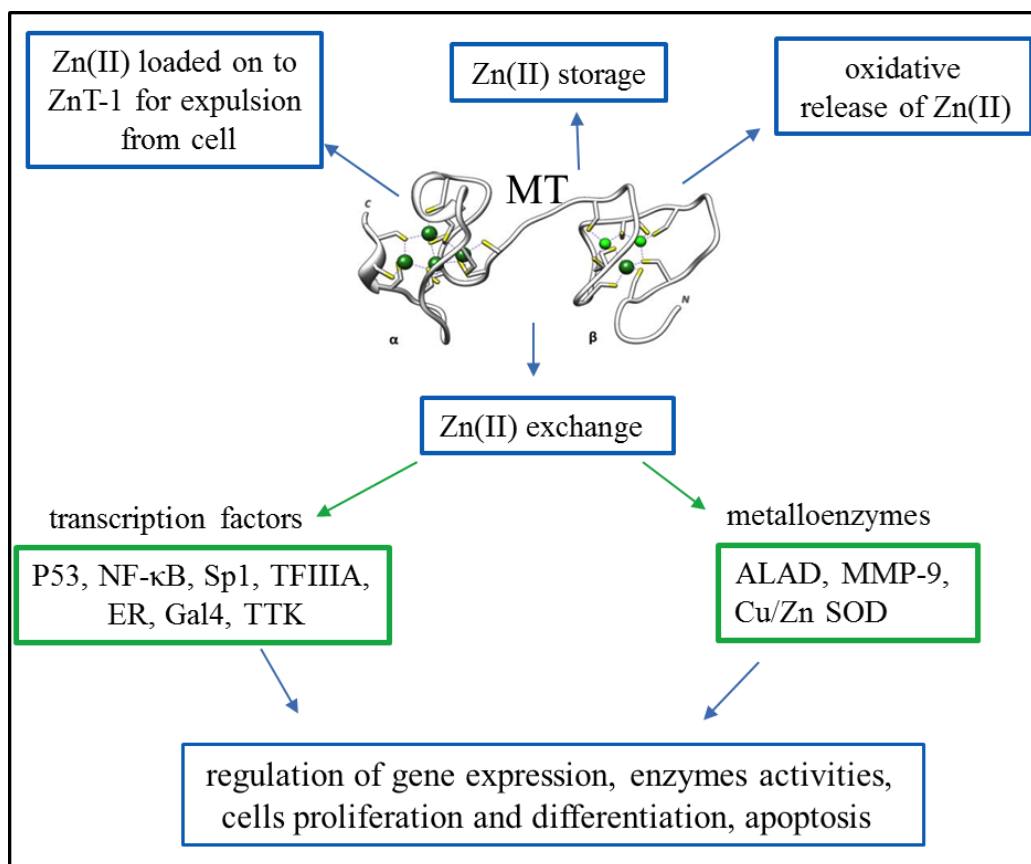
Figure 2. Metallothionein (MT) in zinc trafficking and functions. ALAD, delta-aminolevulinic dehydratase; ER, estrogen receptor; MMP-9, matrix metalloproteinase-9; MT- metallothionein; NF-κB, nuclear factor-kappa B; Cu/Zn SOD, Zn/Cu superoxide dismutase; Sp1, specificity protein 1; TFIIIA, transcription factor IIIA; TTK, tramtrack; ZnT-1, zinc transporter 1.

In mammals, four distinct MT isoforms designated MT-1 through MT-4 exist, whereas they are monomeric proteins, containing two metal-thiolate clusters [143]. In humans, at least 10 to 17 MT genes, clustered on chromosome 16, are functional and encode multiple isoforms of MT-1 (MT-1A, -B, -E, -F, -G, -H, -I, -J, -K, -L, and -X) and one isoform of MT-2 (MT-2A), single genes code for MT-3 and MT-4 [186,187]. Heterogeneity of isoforms results from posttranslational modifications (acetylation) and/or variations in heavy metals content (metalloforms). Isoforms are distributed in various ratios within single tissues and have different rates of degradation [188]. Although the general physicochemical properties of MT isoforms are similar, they have specialized biological functions [185]. The first discovered MT-1/MT-2 are widely expressed isoforms, whose biosynthesis is inducible by a wide range of stimuli, including metals, drugs, and inflammatory mediators [189]. In contrast, MT-3 and MT-4 are metal-non-inducible proteins, with their expression primarily confined to the central nervous system and certain squamous epithelia, respectively. MT-1 through MT-3 have been reported to be secreted, suggesting that they may play different biological roles in the intracellular and extracellular space [190]. Experiments on MT found significant differences in zinc-binding affinities of cysteine clusters [191]. Affinity varies in nanomolar to picomolar range of Zn(II) concentration, i.e. in at least three orders of magnitude [141]. In addition, there were published papers

studying the influence of oxidation on polymerizing of MT and its capacity to bind Zn(II) ions [192,193].

Metallothioneins play a key role in metabolism, transport and storage of heavy metals, particularly Zn(II) [141,194]. Schematic drawing of MT role in Zn trafficking and functions is shown in Fig. 2. When Zn(II) ions get into a cytosol through zinc transporters, it is immediately buffered by MT, thus the free Zn(II) ions level is maintained on very low level, in picomolar to nanomolar range [141]. Due to signalling roles of free Zn(II) ions, MT play an important role in this process because of its level of regulation. Whether zinc(II) level exceeds the buffering capacity of MT, it is being eliminated out of cells by ZnT-1 exporter and subsequently, Zn(II) induces expression of MT and ZnT-1 by binding to transcription factor MTF-1 that binds to metal responsive elements (MREs) which regulate MT expression [163]. It is not surprising that there have been developed several different assays and test to detect and to determine MT [192,193,195-210]

5. ZINC AND ITS INVOLVEMENT IN OXIDATIVE STRESS

Because of zinc omnipresence and importance in the structural maintenance and the activity of a high number of proteins [211], its excess and deficiency might cause a significant alteration in the cell status including oxidative stress through an excessive generation of reactive oxygen species (ROS) [212]. Therefore, zinc homeostasis is critical for cell survival in such a way that zinc concentration can only vary within a narrow range. Despite the inert redox properties of Zn under physiological conditions there is a large number of the indirect oxidative effects which have been attributed to changes in Zn content in cells, tissues and complete living organisms. Oxidative stress as a consequence of ROS generation in Zn deficiency is a recurrent topic in a number of papers (reviewed in [213]). ROS production has been observed in Zn-deficient experimental animals [214-218], as well as in limited Zn cultured eukaryotic/prokaryotic organisms [219-222].

The source of ROS under limited zinc conditions is not clearly understood yet. Several mechanisms have been proposed to explain the role of zinc deficiency in ROS generation in isolated cells as well as in multicellular systems. In general all of these mechanisms imply to recognize that Zn acts as an antioxidant [223-226] since a limited availability of Zn appears to be correlated with an inactivation of the defences against the oxidative stress. A first mechanism considers that low level of Zn induces ROS generation due to the lack of regulation of antioxidant proteins (namely Cu/Zn superoxide dismutase (SOD) or MT) or proteins that indirectly modulate oxidant response in cell (inhibition of *N*-methyl-*D*-aspartate receptor) [227,228]. Secondly, it is also postulated that Zn can compete with Cu or Fe for membrane binding sites therefore a low Zn level makes it unable to compete with active redox ions Cu and Fe [229]. A third mechanism proposes a protection role for Zn towards thiol moieties in proteins which would likely be exposed to oxidation under low concentration of Zn [223]. Finally, the up-regulation of antioxidant genes (transcription factor NF-E2-related-factor 2) is considered as one of the major antioxidant mechanism in which Zn ion appears to be involved [230]. With regard to the first mechanism, the role of Zn consists of the maintenance of adequate level of antioxidant proteins. One of these proteins is metallothionein, a cysteine-rich proteins family

involved in cellular defence against the oxidative stress [231,232] which decreases in some pathological states. It is known that diabetes causes Zn deficiency [233] and in a recent paper it has been reported that OVE26 transgenic type 1 diabetic mouse model shows a significant increase in oxidative hepatic damage [234]. It was observed that Zn supplementation in OVE26 mice allowed a significant reversal of the pathological hepatic changes, including the oxidative effects, through a mechanism of MT up-regulation, thus supporting the protective and antioxidant role of Zn.

On the other hand, not just one response in the activity of antioxidant proteins is obtained as a consequence of low cellular Zn levels. In this regard, ROS production linked to a low available Zn levels, is accompanied by a SOD increased activity as noted in several studies [220,221,235,236]. However a decrease of level and activity of cytosolic SOD1 is observed in the yeast growth under limited Zn conditions [237]. But, in this case after the SOD1 overexpression and repletion of Zn levels oxidative stress was not eliminated. Beside this fact, it has been reported that Pb toxicity in zebrafish (*Danio rerio*) liver does not increase SOD activity as might be expected after an increased ROS generation induced by Pb [238]. This phenomenon results from conformational and functional changes in Cu/Zn SOD, which releases Cu(II) and Zn(II) from the catalytic pocket of SOD provoking enzyme inactivity. From these facts it could be derived that there is no simple linear correlation in the Zn level, antioxidant proteins activity and suppression of the oxidative stress.

In relation to the second mechanism cited above, it is known that two possible pathways could originate interferences in ROS formation. One of them interferes by removing ("pulling effect") active redox metal from its binding site by using a high affinity chelator and the other one involves a replacement ("pushing effect") of the redox-active metal by another inert metal. Both pathways allow prevention of Fenton reaction through the elimination of catalytic activity of redox metal. The role of Zn appears basically related to the displacement of active redox metal thus deactivating its capacity to transfer electrons in a particular environment [239-241] so that, in a Zn deficiency status, this mechanism could not be reached.

The third mechanism mentioned above attributes a Zn antioxidant activity as a result of the protection of thiols which could be accomplished in three possible ways: a) by direct binding of Zn to the thiol moieties; b) by a conformational change in thiol moieties; and c) by chelation of a Zn ions next to thiol moieties which produces a decreased thiol reactivity by a steric hindrance [242]. Zinc binding in protein structures can be disrupted by molecules such as NO, H_2O_2, oxidized glutathione (GSSG), which lead to Zn release, triggering several regulation actions as a consequence of the availability of "free" zinc [243,244]. This process may compromise the activity or function of the protein previously bound to Zn. As indicated, releasing zinc from thiol-Zn bonds by oxidant molecules may or may not affect the activity of the protein including the capacity to bind to DNA, the activation or inhibition of signalling activity in kinase or phosphatase, the nuclear transport of signalling proteins. In addition, Zn release can act directly in modulation of signalling cascades, the regulation of glutathione homeostasis or in the activation or inhibition of kinases and phosphatases (reviewed in [226]).

6. DETERMINATION OF ZINC IN HUMAN BODY

The challenge of the tracing of zinc is to perform the metal speciation and the determination of the chemical equilibrium of the metal in both free form and when complexed with proteins. Numerous analytical techniques have been developed and adapted to qualify and accurately quantify the zinc content in biological matrices (Zn records obtained by common analytical techniques are depicted in Figs. 3A-C). The most common analytical techniques are inductively coupled plasma mass spectrometry (ICP-MS), electrothermal atomic absorption spectrometry (ETAAS) and total reflection X-ray fluorescence (TXRF) because of the high sensitivity and low sample quantity requirements of these methods [245]. Electrochemical methods offer possibilities to be miniaturized and thus employed for development of portable devices for zinc analyses [246].
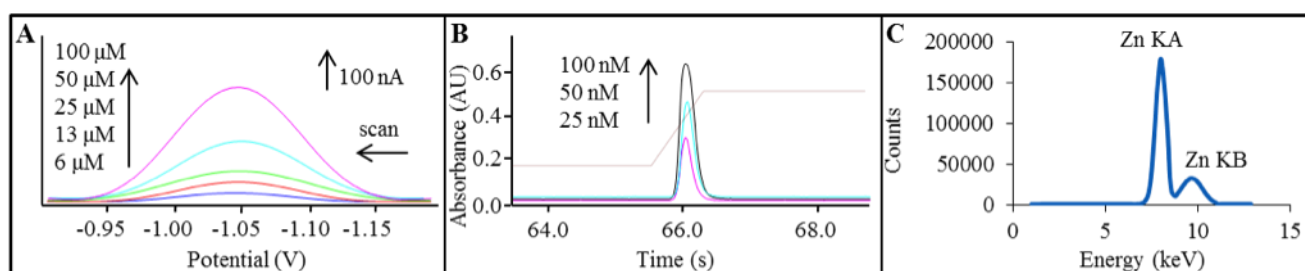
Figure 3. Illustration of Zn records by using various analytical methods. (**A**) Electrochemical record (differential pulse voltammogram) of Zn in 0.2 M acetate buffer (pH = 5) as electrolyte, showing the typical Zn peak at potential of -1.05 V. (**B**) Atomic absorption spectra of Zn, and (**C**) Energy dispersive spectrum of Zn.

6.1. AAS and ICP

ICP-MS has been widely used to detect Zn in biological matrixes such as blood and urine. This technique is very sensitive as well as being accurate and precise. Although there are numerous manuscripts in literature which utilizes ICP-MS to detect Zn in human body, we have focused on five manuscripts. These five manuscripts reported are based on Zn intakes study by a human group.

ICP can be employed as a sequential method for measuring Zn in serum as was shown in [247]. The method is as sensitive as atomic absorption for zinc (sensitivity: 0.11 µmol/l) for patients receiving total nutrition therapy or hemodialysis. A reference interval was established with 34 sera of control subjects (19 men, 15 women) which showed an average zinc, copper and aluminium of 14.5 (S.D. 2.6), 17.3 (S.D. 2.1) and 0.32 (S.D. 0.12) µmol/l, respectively. This method does not require a simultaneous ICP spectrometer and can be performed with 1 ml of serum in a single tube, using a routine sequential ICP spectrometer.

In another study, the performances of a cross-flow nebulizer and a direct-injection nebulizer (DIN) were compared for the determination of zinc in human and urine by ICP-MS [248]. Results obtained by ICP MS using calibration with aqueous standard solutions were found to be in good agreement with those obtained by flame AAS for a batch of real blood plasma and urine samples.

Heitland *et al.* (2006) described the detection of Zn in urine samples of 72 children and 87 adults from two geographical areas of Germany by using inductively coupled plasma mass spectrometry (ICP-MS) with a new octopole based collision/reaction cell. The urine samples were analysed directly after a simple 1/5 (*v/v*) dilution with deionised water and nitric acid with exceptional analytical performance [249]. Goulle *et al.* (2005) utilized inductively coupled plasma mass spectrometry (ICP-MS) to detect Zn in human whole blood, plasma, urine and hair with Rhodium as internal standard [250]. 0.4 ml of the sample was diluted with purified water, acid, triton X100 and butanol. The urine sample results were corrected for creatinine enzymatic activity. Hair samples (25 mg) were acid mineralized after a decontamination procedure and diluted as previously described for biological fluids. Adler *et al.* (2015) developed and validated a simplified sample preparation for the analysis of Zn in whole blood [251]. Analytical measurement range was from 50 to 1500 µg/dL, limit of quantification of 50 µg/dL with coefficient of variation 14%.

Atomic absorption spectrometry (AAS) has been extensively utilized for the determination of Zn in biological samples [252-254]. In literature there are a great number of manuscripts which utilized AAS for analyses of human biological matrixes such as blood and saliva. For instance, ET AAS technique was reported as a suitable approach for the determination of sub-nanogram amounts of Zn in aqueous and blood serum matrices [255]. Burgura-Pascu *et al.* (2007) reported an automated method for the determination of zinc in human saliva by ET AAS after on-line dilution of samples with a significant reduction of sample consumption per analysis (<0.4 ml including the dead volume of the system). Zinc levels in saliva samples from 44 healthy volunteers, 15 male and 29 female, with ages between 20 and 51 years (mean 30.50 ± 9.14 years) were in the range $22 - 98 \ \mu gl^{-1}$ (mean of 55 ± $17 \mu gl^{-1}$), similar to some and different from others reported in the literature. It was found that zinc values for male were statistically higher ($p = 0.006$) than for female [256].

6.2. Electrochemistry

Electrochemistry is an advantageous analytical tool, which is cost effective, portable and fast. It has been widely employed in biological matrixes [203,257-259] and pharmaceutical [260] due to its continuance, sensitivity, reproducibility and selectivity towards many target analytes [261]. Stripping analysis in particular is a powerful and simple tool to determine trace target metal species. It has been widely used in environmental or clinical samples due to its inherent sensitivity.

Tripathi *et al.* (2001) used anodic stripping voltammetry (ASV) with the use of a hanging drop mercury electrode and with complementary atomic absorption spectroscopy to quantify zinc in infants [262]. In the study the whole blood samples of Mumbai and Hyderabad children was determined. 576 blood samples of children (3 – 6 years old) were collected during 1996 – 1998 and analyzed for Zn together with other biometals. Potentiometric anodic stripping voltammetry can be employed also after sample digestion as was shown in human blood samples [263]. Using this method, the levels found in the whole blood sample in a group of 82 people were of Zn: 6.95 ± 1.08 µg/ml. Kruusma *et al.* (2005) described three different electroanalytical techniques for the determination of zinc in blood [264]. The direct determination in diluted blood via anodic stripping voltammetry at glassy carbon and the use of

nafion-coated glassy carbon mercury electrodes were reported to lack the necessary sensitivity whereas an acoustically assisted double extraction followed by sono-ASV using a glassy carbon electrode was found to be rapid, reliable and sensitive. Crew *et al.* (2008) on the contrary reported in human sweat, the development of a novel electrochemical assay for Zn, which involves the use of disposable screen printed carbon electrodes (SPCEs) [265]. The method was applied to the determination of the analyte in sweat from 10 human volunteers. The concentrations were between 0.39 and 1.56 µg/mL, which agrees well with previously reported values.

Other possibilities of electrochemical determination of heavy metals are ion-selective electrodes, which define the selectivity of of a sensor via selective complex formation with the analyte [266]. Several ion-.selective elecrodes have been developed and tested for Zn(II) determination in biological sample, for example [267-270]. However these electrodes often suffer from poor selectivity and robustness for determination in biological samples. The last attempt is polymeric Zn(II) selective electrode based on 2,6- diacetylpyridinebis(benzenesulfonylhydrazide) ligand have been prepared and tested for Zn(II) quantification in wastewaters. The results were in good agreement with AAS and the authors steted, that [271].

7. CONCLUSIONS

It is clear that zinc does play a substantial role in metabolism, organism protection and its deficiency is involved in the development of different diseases as low levels found in neurodegenerative diseases such as Alzheimer's disease (AD) or Parkinson's, liver failure, renal dysfunction, heart problems and different types of cancer. However, the direct effects on prevention or treatment of these pathological states have to be further elucidated. Despite this fact, an adequate intake therefore is necessary to sustain proper metabolic and tissue functions.

On the other hand, the discovery and determination of Zn biomarkers might be useful as new biological insights applied in prevention, molecular diagnosis, prognosis and treatment of different pathological states identifying patients at high-risk status of disease. Zn is now being extensively investigated for utilization in a wide area ranging from therapeutic activity, drug and gene delivery, to nanotechnology with promising outcomes, and thus in can be stated that zinc will attract the research attention in the future.

Conflict of interest
The authors have declared no conflict of interest.

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