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Session 2: Micronutrients and the immune system
Zinc, metallothioneins and immunosenescence

E. Mocchegiani[1]*, M. Malavolta[1], L. Costarelli[1], R. Giacconi[1], C. Cipriano[1], F. Piacenza[1], S. Tesei[1], A. Basso[1], S. Pierpaoli[1] and F. Lattanzio[2]

[1]*Centre Nutrition and Ageing*

[2]*Scientific Direction, Italian National Research Centres on Ageing (INRCA), Via Birarelli 8, 60121 Ancona, Italy*

Ageing is an inevitable biological process with gradual and spontaneous biochemical and physiological changes and increased susceptibility to diseases. The nutritional factor, zinc, may remodel these changes with subsequent healthy ageing, because zinc improves the inflammatory/immune response as shown by *in vitro* and *in vivo* studies. The intracellular zinc homeostasis is regulated by buffering metallothioneins (MT) and zinc transporters (ZnT and ZIP families) that mediate the intracellular zinc signalling assigning to zinc a role of 'second messenger'. In ageing, the intracellular zinc homeostasis is altered, because high MT are unable to release zinc and some zinc transporters deputed to zinc influx (ZIP family) are defective leading to low intracellular zinc content for the immune efficiency. Physiological zinc supplementation in the elderly improves these functions. However, the choice of old subjects for zinc supplementation has to be performed in relation to the specific genetic background of MT and IL-6, because the latter is involved both in MTmRNA and in intracellular zinc homeostasis. Old subjects carrying GG genotypes (C– carriers) in the IL-6–174G/C locus display high IL-6, low intracellular zinc content, impaired innate immunity and enhanced MT. Old subjects carrying GC and CC genotypes (C+ carriers) display satisfactory intracellular zinc content, adequate innate immunity and are more prone to reach longevity. Zinc supplementation in old C– carriers restores natural killer cell cytotoxicity and zinc status. The genetic variations of the IL-6 – 174G/C locus when associated with those of the MT1A + 647A/C locus are useful tools for the choice of old people for zinc supplementation.

Zinc supplementation: Metallothioneins: Zinc transporters: Zinc signalling: Inflammation: Immune response: Longevity: Ageing

Ageing is accompanied by gradual biochemical and physiological changes including increased susceptibility to diseases and adverse environmental conditions, and loss of mobility and agility. The inability of an organism to adjust to the changes may lead to some degenerative age-related disease, and, to address this, the 'remodelling theory of aging' has been proposed[1]. Various nutritional factors can affect age-associated changes. Approximately 40 micronutrients are essential components of the diet. The dietary intake of essential macro- and micronutrients is usually inadequate in the elderly[2] and several factors contribute to this deficiency. Firstly, the poor socio-economic status of many elderly individuals may lead to a greater consumption of inexpensive foods deficient in micronutrients (e.g. carbohydrates)[3]. Nutrient deficiency is then exacerbated by loss of appetite, lack of teeth, intestinal malabsorption and decreased energy requirement, all of which can lead to frailty, disability and functional dependence[4]. The deficiency of macro- and micronutrients in the elderly is strictly related to global impairment of immune functions, metabolic harmony, antioxidant defence by external noxae and involved in mitochondrial decay[5]. Recent longitudinal studies of daily dietary intake in human centenarians (successful ageing) showed that an adequate

Abbreviations: DTH, delayed type hypersensitivity; IFN-γ, interferon-γ; MT, metallothionein; NK, natural killer; NO, nitric oxide.

***Corresponding author:** Dr E. Mocchegiani, fax +39 071 206791, email e.mocchegiani@inrca.it

consumption of micro- and macronutrients leads to good performance in several immune functions, to metabolic compensation, for the preservation of antioxidant activity and mitochondria functionality[6]. Therefore, nutritional factors may play a pivotal role in achieving healthy ageing and longevity. Among them, zinc is one of the most relevant nutritional factors in ageing, because it affects the immune response, metabolic harmony and antioxidant activity, leading to a healthy state[7]. On the cellular level, zinc is essential for proliferation and differentiation and is involved in signal transduction and apoptosis[8]. The cells depend on a regular supply of zinc and make use of a complex homeostatic regulation by many proteins, but the plasma pool, which is required for zinc distribution, represents <1 % of the total body content[9]. Despite its important function, the body has only limited zinc stores that are easily depleted and cannot compensate longer periods of zinc deficiency. Additionally, pro-inflammatory cytokines mediate changes in hepatic zinc homeostasis during infections, leading to sequestration of zinc into liver cells and subsequently to hypozincaemia[10]. Alterations in zinc uptake, retention, sequestration, or secretion can quickly lead to zinc deficiency and affect many zinc-dependent functions in virtually all tissues, and in particular the immune response.

Taking into account that zinc homeostasis is regulated by metallothioneins (MT) during an inflammatory/immune response[11], the interrelationship between zinc and MT is crucial in ageing in order to prevent disabilities due to age-related diseases. The maintenance of zinc homeostasis is also under the control of multiple transmembrane transporters (named zinc transporters) evolved to modulate the storage, efflux and uptake of zinc in response to its availability[12]. In this study, we review the role of zinc and MT on inflammatory/immune response in ageing and in successful ageing with a focus on the possible effect of zinc supplementation upon the immune system in old mice and elderly individuals carrying specific genetic variants in MT and IL-6 genes which, in turn, affect intracellular zinc homeostasis[9]. The role played by zinc signalling and some zinc transporters is also reported and discussed.

Zinc-metallothioneins and ageing

MT are a group of low-molecular-weight metal-binding proteins that have high affinity for zinc (k_d (constant of dissociation) $1{\cdot}4 \times 10^{-13}$ M)[13]. MT exist in different isoforms characterized by the length of amino acid chain: isoforms I, II, III and IV mapped on chromosome 16 in man and on chromosome 8 in mice with complex polymorphisms[9]. The more common isoforms are I and II; the isoform III is a brain-specific member and the isoform IV is restricted to squamous epithelia. MT contain 20 cysteines, all in reduced form, and bind seven zinc atoms through mercaptide bonds that have the spectroscopy characteristics of metal thiolate clusters[13]. MT distribute intracellular zinc as zinc undergoes rapid inter- and intracluster exchange[13]. The redox properties of MT and their effect on zinc in the clusters are crucial for the protective role of MT in the presence of ionizing and UV radiations, heavy toxic metals (mercury and cadmium), lipid peroxidation, reactive oxygen species, oxidative stress caused by anticancer drugs and conditions of hyperoxia[14]. This protective role of MT has been especially studied in young–adult MT knockout mice (null mice) for short periods of exposure to toxic metals or in the presence of zinc excess or zinc deficiency[15]. Therefore, the protective role of MT is evident in transient stress conditions, as it may occur in the young–adult age, in which the chronic status (by stress or inflammation) is a rare event[9]. In contrast, this role may be questionable in ageing, because the stress-like condition and the inflammation by high IL-6 are chronic[9]. Since IL-6 affects MT gene expression[16], these proteins may turn off from protective to harmful agents in ageing following the 'Antagonistic Pleiotropy Theory of Ageing'[17]. Despite MT increase in ageing, a limited release of zinc by MT occurs suggesting one of the possible causes of the impaired immune response[9]. In contrast, in the presence of lower stress and inflammatory condition, as it occurs in centenarians, MT production is low, coupled with satisfactory intracellular zinc ion availability[16]. Since IL-6 acts through its sub-unit receptor gp130, the relative lower gene expression of gp130 in centenarians[18] may imply that a quota of IL-6 is inactive in very old age. As a result, the satisfactory immune performance, metabolic harmony and antioxidant activity allow a good healthy status in centenarians[16]. Therefore, the interrelationships among stress/inflammatory/immune status, MT and zinc are pivotal in order to achieve successful ageing. However, this role remains to be clearly established taking also into account that MT may play different roles in different organs. In this regard, recent findings in cardiac-specific MT transgenic mice suggest that the expression of MT in cardiocytes may alleviate ageing-induced cardiac contractile defects and oxidative stress prolonging the lifespan[19]. In addition, Daf-2 (gene of insulin receptor-like protein-2) mutant nematodes, other than a longevity phenotype, display an altered expression of MT which, in turn, seem to interact with the insulin signalling pathway[20]. Therefore, even if the specific function of MT in ageing is still a matter of discussion, these last reports, associated with recent findings on the possible role played by MT in modulating energy metabolism[21], strongly suggest that MT is pivotal for maintaining the health status. On the other hand, polymorphisms of MT1A (A/C at position +647) are involved in successful ageing[22].

Zinc-metallothioneins and inflammatory/immune response in ageing

For a prompt immune response against stressor agents and inflammation, macrophages produce some cytokines, such as IL-1, IL-6, interferon (IFN)-α, TNF-α, which in turn provoke a new synthesis of MT in the liver, but at the same time, an alteration in the zinc status[9]. These findings clearly suggest the existence of interplay between MT and the immune system. MT act both as a reservoir of zinc during zinc deficiency and as a zinc buffering protein in the presence of excessive amount of zinc in order to prevent zinc toxicity[15]. Therefore, MT are protective agents

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with also the task to prevent zinc deficiency during an inflammatory status. Under inflammatory conditions, MT in the extra-cellular environment may support the beneficial movement of leucocytes to the site of inflammation representing a 'danger signal' for the immune cells and modifying the character of the immune response when cells sense cellular stress. However, high MT produced in chronic inflammation may alter the normal chemotactic response that regulates leucocyte trafficking[23]. Taking into account that zinc ions attract leucocytes by promoting the chemotactic response[24], high MT might thus be dangerous for the immune response during chronic inflammation. Moreover, (1) the existence of high MT and low zinc ion availability in the atrophic thymus from old mice[25], (2) the presence of high MT in lymphocytes from old people and Down's syndrome subjects (premature ageing) coupled with impaired innate immunity[16] and (3) the occurrence of atrophic thymus in young stressed mice over-expressing MT[26], further suggest this dangerous role played by MT in immune response during ageing. Additionally, elevated levels of extra-cellular MT, as found in chronic inflammatory sites, can cause a dramatic decrease in cytotoxic T lymphocyte activity against allogeneic target cells, reduce the proliferative response of CTLL-2 (IL-2 dependent cytotoxic T-lymphocyte cell line) cells to cytokines, and decrease the level of MHC Class I and CD8 molecules[27]. Or paradoxically, high levels of MT induced by antigenic stimuli may allow an iper-activation of the immune system with subsequent deleterious effect, as it occurs in revertant CD4 T-cells from older individuals[28]. Therefore, high MT may be considered as a consequence of the intracellular zinc dyshomeostasis further supporting its task as a specific 'danger signal' for the efficiency of the immune system in ageing. Anyway, high MT may be harmful in ageing. This role may be largely due to the increased zinc influx within the cells during acute or chronic inflammation that, in turn, provokes increased MT with concomitant decrement in intracellular labile zinc, because a majority of the zinc ions are buffered by MT[29]. This phenomenon is observed in old people (aged up to 70–75 years.), but not in very old people (>80 years), in whom both MT and intracellular labile zinc are low perhaps due to lower dietary zinc intake or cellular senescence phenomena[29]. Subsequently, the immune efficiency is diminished or altered in ageing and in very old age. This effect may be worsened by the fact that MT are not efficient donors of zinc in ageing[16]. On the other hand, high MT induce down-regulation of many other biological functions related to zinc, such as metabolism, gene expression and signal transduction[9].

However, the limited capability of MT in zinc release is still an unresolved problem in ageing, especially with regard to the precise mechanism involved. The zinc release from MT under oxidative stress conditions is accompanied by more MT disulfide bond formation[30]. But, an intriguing point is that also nitric oxide (NO) provokes the zinc release by MT, via s-nitrosylation of MT cysteines (i.e. the transfer of an NO group to cysteine sulfhydryl groups on MT molecule), and Zn^{2++} release is sufficiently mild to allow the reconstitution of MT through Zn^{2++} rebinding[31]. During inflammation, hepatocytes respond to cytokines by up-regulating inducible NO synthase, which generates a large amount of NO from arginine with concomitant enhanced MT[32]. NO promotes zinc release from MT, which in turn may repress inducible NO synthase in an autocrine way[31]. However, despite inducible NO synthase increases in ageing, the release of zinc by MT is very limited. NO donors and zinc fluorescent probes are useful tools in order to study the zinc release from MT and to evaluate the intracellular labile zinc in ageing. Using a methodology recently developed in our laboratory[33], the NO-induced release of zinc can be preserved at least in non-agenarians carrying MT1A polymorphism favourable to the longevity[22]. Moreover, a flow cytometric assay for the measurement of intracellular labile zinc shows that the intracellular concentration of labile zinc in resting cells were estimated to be 0·17 nM in monocytes and 0·35 nM in lymphocytes (CD4+)[34]. The combination of these two novel methodological procedures will permit to study in depth the cause of limited zinc release from MT in ageing and, at the same time, to evaluate the amount of intracellular labile zinc. Anyway, a limited zinc release from MT exists in ageing with an emphasis after 70 years of age[29]. The recent discovery of a novel polymorphism of MT (−209A/G MT2A) may indirectly support this assumption. Old subjects carrying AA genotype display high MT, low intracellular zinc ion availability, enhanced IL-6 and impaired innate immune response with subsequent possible risk to develop atherosclerosis and diabetes type-2[35]. Therefore, MT might have a different role in immunosenescence, fitting thus with the concept that several genes/proteins that increase fitness early in life may have negative effects later in life[17].

Zinc transporters, zinc signalling, inflammatory/immune response and ageing

Zinc transporter proteins also appear to be specifically involved in regulating cellular zinc homeostasis via influx, efflux or vesicular sequestration with a task in maintaining intracellular zinc concentration in a narrow physiological range in order to avoid cellular zinc toxicity or deficiency when extra-cellular zinc concentration changes[12]. Two families of zinc transporters have been identified. The ZnT family decreases cytoplasmic zinc concentration by secretion, sequestration or efflux, whereas the ZIP family increases cytoplasmic zinc influx or release of stored zinc[12]. Therefore, the balance of zinc transporter families is fundamental in maintaining an optimal intracellular zinc homeostasis as well as the zinc signalling. In this context, ZIP7 releases Zn from the endoplasmic reticulum controlling tyrosine phosphorylation[36], and lysosomal ZIP8 is required for IFN-γ expression in T-cells[37]. ZIP6 is implicated in the zinc signalling required for revertant CD4 T-cell proliferation, via activation of NF-κB and subsequent pro-inflammatory cytokine productions[28]. The zinc signal via ZIP6 is also responsible for MT2A induction which, in turn, mediates a negative feedback to down-regulate this signal. The relevance of the zinc signals in regulating inflammatory signalling, via NF-κB, was also observed in monocytes treated with lipopolysaccharide[38],

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suggesting the relevance of zinc transporters and zinc signalling in modulating the immune response, especially in ageing because of the likely reduced zinc dietary intake and intestinal malabsorption[9].

However, a paucity of data exists regarding the role played by zinc transporters in ageing. After an increase from the birth up to the adult age in some tissues, significant decrements of both ZnT and ZIP families in peripheral leucocytes from elderly women have been observed, in particular the sub-types ZnT1 and ZIP1[39]. Taking into account that ZIP family increases cytoplasmic zinc influx[12], an intriguing point is that IL-6 up-regulates the ZIP14 gene expression in the liver, which is in turn responsible for the hypozincaemia that accompanies the acute phase response to inflammation and infection[10]. Since chronic inflammation by high IL-6, hypozincaemia and risk of infections are common in old age[16], the possible alterations of the zinc transporters in ageing coupled with the inability of MT in zinc release, might induce synergistic deleterious effects on immune efficiency with the subsequent emergence of some age-related diseases.

Rationale for zinc supplementation in ageing: *in vitro* studies

Since the crude zinc balance is negative in old mice and human individuals[40], zinc supplementations in old mice and in the elderly have been carried out in order to restore the immune efficiency. The scientific rationale for *in vivo* zinc supplementation finds consistent support by *in vitro* data in immune cells. When peripheral blood mononuclear cells are stimulated with zinc, IL-1, IL-6 and TNF-α, soluble IL-2 receptor and IFN-γ are released[41]. However, the effect of zinc on monocytes may depend on external stimulation. In fact, zinc inhibits lipopolysaccharide-induced TNF-α and IL-1-β release from both primary human monocytes and monocytic cell lines through the inhibition of cyclic nucleotide phosphodiesterase activity[41], suggesting that zinc may also display some anti-inflammatory properties. The dose of zinc used is also a critical variable. In a serum-free culture medium, the concentration of zinc ≥100 μM stimulates monocytes, but prevents T-cell activation, perhaps owing to a lower intracellular zinc content in T-cells than in monocytes[41].

Treatment with zinc *in vitro* generally also displays beneficial effects on cell survival, but the effect largely depends upon the cell type and the dose of zinc used. It seems that both apoptosis prevention and induction are mediated by pathways involving zinc and/or zinc-dependent enzymes[8]. Therefore, the modulation of the intracellular zinc homeostasis plays a key role not only in preventing apoptosis, when oxidative stress or chronic inflammation are low, but also in inducing apoptosis especially when oxidative stress and cellular damage is high in order to down-regulate the immune response and to eliminate virally infected or malignant cells. Induction of apoptosis by zinc signalling in damaged cells, via activation of p53 pathway, is evident in young–adult age and in very old age[42], perhaps owing to a preserved regulation of zinc homeostasis in both classes of age[16].

Experiments in thymocytes also support this point of view, since media supplemented with zinc from 50 up to 150 μM prevents old thymocyte apoptosis induced by dexamethasone or serum deprivation[43], whereas the direct introduction of free zinc, as zinc-pyrithone, inside thymocytes provokes apoptosis because of inducing permanent oxidative stress and irreversible damage[44], thus activating pro-apoptotic pathways. Therefore, zinc supplementation, not exceeding the physiological dose, may be of benefit in ageing either in preventing apoptosis of undamaged immune cells or in reducing the inflammation with a possible prevention of the appearance of age-related diseases.

Effect of zinc supplementation on inflammatory/immune response in ageing

Old mice

Old literature reports that a physiological zinc supplementation in the diet throughout the life span in adult rodents prevents some age-related cell-mediated immune modifications[45]. Recently, a physiological zinc supplementation (18 μg/ml Zn^{2++} in the drinking water for 1 month) in old mice induced thymus re-growth and its endocrine activity coupled with an improvement of peripheral NK (natural killer) cell cytotoxicity[40,46]. Zinc supplementation (300 mg/kg for 25 d) in aged mice improved thymopoiesis, as assessed by increased total thymocyte numbers[47]. The improved thymic output was mediated in part by reducing the age-related accumulation of immature CD4(−)CD8(−)CD44(+)CD25(−) thymocytes, as well as by decreasing the expression of stem cell factor, a thymosuppressive cytokine[47]. Moreover, old mice treated with zinc in drinking water daily from the pre-senescent age (12–14 month of age) display a significant increment of the mean lifespan when compared to controls[40]. The increased mean lifespan is largely due to significant decrement of deaths from cancer and infectious diseases in the period between 24 and 25 months of age[40]. Of interest, nude and neonatal thymectomized mice, which display negative crude zinc balance and a very short life due to thymus absence, also show increased rate of survival after zinc supplementation[48]. Taking into account that the liver extrathymic T-cell pathway is prominent in old, nude and neonatal thymectomized mice in order to compensate the thymic failure[9], it is thus evident that zinc also affects the liver extrathymic T-cell pathway with subsequent good peripheral immune performance, especially liver NK cells bearing T-cell receptor on their membrane surface (NKT cells) bearing T-cell receptor γδ[9]. Preliminary data from our laboratory have also shown that MT null mice display shorter survival in comparison with mice over-expressing MT, suggesting the pivotal role played by MT for longevity.

The elderly

With regard to the elderly, inconsistent data exist on the beneficial effect of zinc supplementation on the immune efficiency, due to different doses and duration of zinc treatment. Although zinc was used at the dose recommended by

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Table 1. Zinc supplementation studies in human elderly and old mice: effect upon the immune functions

	Subjects	*n*	Intervention*	Effect	Reference
Elderly	Institutionalized, >70 years	15 (C) 15 (Z)	100 mg Zn as sulphate for 1 month	Increased T-cell numbers, DTH, and response to tetanus vaccine	(56)
	Anergic to DTH, 64–76 years	5 (Z)	55 mg Zn as sulphate for 4 weeks	Improved DTH	(59)
	Free-living, 60–89 years	36 (P) 36 (Z,15) 31 (Z,100)	15 or 100 mg Zn as acetate for 3 months	No effect on DTH or *in vitro* lymphocyte proliferation	(61)
	Zinc-deficient males, 65–78 years	8 (Z)	60 mg Zn as acetate for 4·5 months	Increase in DTH	(58)
	Free-living, 60–89 years	24 (P) 20 (Z,15) 19 (Z,100)	15 or 100 mg Zn as acetate for 12 months	Negative effect on DTH and NK cell activation only after 3 months	(51)
	Institutionalized, 73–106 years	44 (P)/(Z) crossover	20 mg Zn as gluconate for 8 weeks	Increased thymulin activity	(50)
	Zinc deficient, 50–80 years	13 (Z)	30 mg Zn as gluconate for 6 months	Increased thymulin activity, IL-1, DTH	(60)
	Institutionalized, 64–100 years	190 (C) 160 (Z)	90 mg Zn as sulphate for 60 d or 30 mg as sulphate for 6 months	No effect on antibody response after influenza vaccination and no effect in taste acuity	(62,63)
	Institutionalized, ≥65 years	30 (P) 28 (Z)	25 mg Zn as sulphate for 3 months	Increase in CD4+DR+ T cells and cytotoxic T-cells	(53)
	Free-living, 65–82 years	19 (Z)	10 mg Zn as aspartate for 7 weeks	Reduced levels activated T helper cells and basal IL-6 release from PBMC, improved T-cell response	(55)
	Institutionalized	25 (P) 24 (Z) 6 (P) 6 (Z)	45 mg Zn as gluconate for 12 months, 45 mg Zn as gluconate for 6 months	Reduced incidence of infections increased IL-2 mRNA in response to *ex vivo* stimulation with PHA	(60)
	Healthy, 55–70 years	31 (P) 28/34 (Z)	15/30 mg Zn as gluconate for 6 months	No effect on markers of immunity (NK cells) or inflammation (CRP) Only increased ratio CD4/CD8	(54)
	Healthy elderly and elderly with bronchopneumonia (65–85 years)	15 healthy (Z) 10 with bronchopneumonia (Z)	12 mg Zn as sulphate for 1 month	Increased NK cell cytotoxicity and active thymulin in both groups	(64)
	Healthy, 60–84 years with plasma zinc levels (≤10·5 μM)	110 (Z)	10 mg Zn as aspartate for 7 weeks	Increased NK cell cytotoxicity	(48,68)
Old mice	Male Balb/c	10 (C) 10 (Z)	18 μg Zn as sulphate (22 mg/l in drinking water)	Thymus re-growth and functional recovery; increased NK cell cytotoxicity; significant decrement of deaths due to cancer and infection	(40)
	C57Bl/6 mice	12 (C) 12 (Z)	117 mg/kg of Zn in the enriched food versus 39 mg/kg of Zn (control food)	Increased number of thymic lymphocytes without significant changes in CD4/CD8 thymocyte subsets; increased thymulin levels	(46)
	Male Balb/c	50 (C) 50 (Z)	18 μg as zinc sulphate	Increased mean survival	(40)
	C57Bl/6 mice	8 (C) 8 (Z)	300 mg/kg of Zn for 25 d	Improved thymic output	(47)

DTH, delayed type hypersensitivity reaction; (C), control group without supplementation; (P), placebo; (Z), zinc supplementation; PHA, phytohaemagglutinin; PBMC, peripheral blood mononuclear cells; NK, natural killer; CRP, C-reactive protein.
*The values are given as elemental zinc.

RDA (10–25 mg/d with different length of treatment; Table 1) in the majority of the studies, Prasad *et al.*[49] and Boukaiba *et al.*[50] have found an increment of thymulin activity and improvement in response to skin-test antigens and taste acuity; Bogden *et al.*[51] have reported some benefit exclusively for increased lymphocyte mitogen proliferative response; Cakman *et al.*[52] have found enhanced IFN-γ production by leucocytes; Fortes *et al.*[53] report an increased number of cytotoxic T lymphocytes; Hodkinson *et al.*[54] describe no effect on some markers of immunity (natural killer cells) or inflammation (C-reactive protein), but only increased the ratio of CD4/CD8 T lymphocytes at month 6; Kahmann *et al.*[55] report reduced levels of activated T-cells and basal IL-6 release from peripheral blood mononuclear cells and improved T-cell response. Using higher doses of zinc, 40–220 mg/d with different lengths of treatment (Table 1), Duchateau *et al.*[56] and Sandstead *et al.*[57] have observed an improvement in response to skin-test antigens and taste acuity; an improved delayed type hypersensitivity reaction has been also found in a limited number of subjects by Cossack[58] and by Wagner *et al.*[59]; Prasad *et al.*[60] found improved IL-2 mRNA. Other studies[61–63] report no effects on various immune functions after zinc supplementation, perhaps due to high dose of zinc used for too long a period (Table 1).

From all these studies, a physiological dose of zinc applied for a long period or high doses of zinc for short periods might induce limited effects on the immune response, perhaps due to zinc accumulation in various organs and tissues with subsequent toxic effect of zinc upon the immune functions[9]. In this context, it is also useful to remember that high doses of zinc trigger apoptosis of the immune cells in the presence of high-oxidative stress and inflammation[8]. Therefore, caution is advised for the management of zinc supplementation with the suggestion to perform the trial for short periods and on alternate cycles only. In our experience, zinc treatment (15 mg Zn^{2++}/d for 1 month) in Down's syndrome subjects, the elderly and old infected patients restores thymic endocrine activity, lymphocyte mitogen proliferative response, CD4(+) cell number, NK cell cytotoxicity and DNA repair, as well as thyroid hormones turnover. At the clinical level, significant reductions of relapsing infection occur in Down's syndrome subjects, the elderly and old infected patients[64].

An intriguing point is the increment of zinc transporters after zinc supplementation. Elderly women treated with 22 mg zinc gluconate/d for 27 d display significant increments of ZnT1 gene expression in peripheral leucocytes[39]. Such increments of ZnT1 have been also observed in human lymphoblastoid cells adding *in vitro* 50 or 100 μmol/l zinc[39], further suggesting the relevance of zinc supplementation in affecting the gene expression of zinc transporters and, consequently, the correct maintenance of intracellular zinc homeostasis. Such a mechanism might be important for restoring ZnT8 gene expression in pancreatic vesicles being involved in the aetiology of type-2 diabetes[65].

Since zinc also affects the cytotoxicity of liver NKT cells bearing T-cell receptor γδ (extrathymic T-cell pathway) with higher production of IFN-γ in old mice[66], the presence of satisfactory zinc ion bioavailability coupled with increased NKT cell cytotoxicity and enhanced IFN-γ production in centenarians with respect to the elderly[67] strengthens the pivotal role of zinc supplementation in maintaining or improving the global immune response (thymic and extrathymic T-cell pathways) and in fighting oxidative stress and inflammation.

However, since zinc also affects MT gene expression[13], the question arises whether zinc supplementation in old age may further increase MT, causing possible major harmful effects (i.e. still major limited zinc release by MT). This fact may be avoided, because zinc lowers the inflammation and, as such, MT can still be able to release zinc with subsequent good immune performances[11]. Therefore, the potential harmful effect of MT may be excluded during physiological zinc supplementation in ageing.

Zinc supplementation in the elderly on the basis of genetic background

One possible cause of the discrepancy existing in the literature on the effect of zinc supplementation on the immune response in the elderly may be the choice of old subjects who effectively need zinc supplementation in strict relationship with dietary habits and inflammatory status. This assumption is supported by the discovery that old subjects carrying GG genotypes (termed C− carriers) in the IL-6 −174G/C locus display increased IL-6 production, low intracellular zinc ion availability, impaired innate immune response coupled with enhanced MT[68]. By contrast, old subjects carrying GC and CC genotypes (termed C+ carriers) in the same IL-6 −174 locus displayed satisfactory intracellular zinc as well as innate immune response. But, the more intriguing finding is that male C+ carriers are more prone to reach centenarian age than C− carriers. Therefore, old C− carriers are likely to benefit more from zinc supplementation than old C+ carriers. The distribution of IL-6 −174 genotype is very different among various European countries with large differences between Northern and Southern European countries[69]. Zinc supplementation in old C− subjects restores NK cell cytotoxicity to values present in old C+ carriers and considerably improves both zinc status, assessed by the percentage increment of granulocyte Zn[68], and stress response, assessed by the percentage increment of MT protein as well as Clusterin/apo J and other proteins related to oxidative stress and inflammation[48]. When the genetic variations for IL-6 polymorphism are also associated with the variations of MT1A+647A/C gene, the plasma zinc deficiency and the altered immune response is more evident[69], suggesting that the genetic variations of IL-6 and MT1A are very useful tools for the identification of old people who effectively need zinc supplementation. These results open the hypothesis that the daily requirement of zinc might be different in elderly harbouring a different genetic background. Such a role played by the genetic background on the beneficial effect of zinc supplementation is also evident in keeping the pro-inflammatory cytokine and chemokine productions better under control[69] as well as in reducing the gene expression of genes related to the inflammatory status, such as IL-1

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Table 2. Effect of zinc supplementation in the elderly in accordance with genetic background and zinc status

	Parameter	Effect of the zinc supplementation	General causes of variability observed
Zinc status[48,70]	Plasma zinc	↑	Plasma zinc levels before supplementation, inflammatory status, country (dietary habits), IL-6 −174 and MT1A +647 polymorphisms
	Plasma zinc/albumin	↑	Plasma zinc levels before supplementation, inflammatory status, country (dietary habits), IL-6 −174 and MT1A +647 polymorphism
	Labile intracellular zinc	↑↑	IL-6 −174 polymorphism, country (dietary habits)
	MT	↑	IL-6 −174 and MT1A +647 polymorphisms, country (dietary habits),
	Nitric oxide-induced release of zinc	↑↑	IL-6 −174 polymorphism, country of origin (dietary habits)
	Granulocyte zinc	↑↑	IL-6 −174 polymorphism, country (dietary habits)
	MT glutathionylation	–	–
Stress-related proteins[48,68]	Poly(ADP-ribosyl)ation capacity	↑	Plasma zinc levels before supplementation and increase of plasma zinc after supplementation
	Reactive oxygen species production	↓	Plasma zinc levels before supplementation, age of donors
	ApoJ plasma	–↑	IL-6 −174 polymorphism, country (dietary habits)
	Genes involved in nitrosative stress (ATF2, CSF2, FOS, ICAM1, JUN, LTA, CCL2, SELE, VCAM1, inducible nitric oxide synthase, TNF and NFκB1)	↓	–
	Total intracellular carbonyl levels	↓	–
	MsR activity and protein expression	↑↑	Country (dietary habits)
	Chymotrypsin-like peptidase activity of proteasome and 20S protein expression	↑	–
	Chaperone (heat-shock protein 72) protein levels	–↑	–
	Chaperone (heat-shock protein 72) inducibility	↑↑	–
			–
Antioxidant plasma enzymes[48]	Plasma SOD	↑	–
	Erythrocyte SOD	↑	–
	Catalase	↓	–
	Glutathione peroxidase	↓	–
Thymic output[48]	T-cell receptor excision circles	↓↑	Age, gender, country (dietary habits), plasma zinc at baseline
Senescence and apoptosis[48]	Telomere length	–↑	Country (dietary habits)
	Early spontaneous apoptosis	↓	P53 codon 72 polymorphism
	Late apoptosis	↓	
	Oxidative stress-induced apoptosis	↓	
	Mitochondrial membrane depolarization during spontaneous and dRib-induced apoptosis	↓	
	Cell cycle	–	
Plasma cytokines/ chemokines[69]	IL-6, IL-8 and MIP-1α	–↑	Gender, country (dietary habits), plasma zinc at baseline, IL-6 −174 and MT1A +647 polymorphism
	MCP-1 and RANTES	–	Gender, country (dietary habits), plasma zinc at baseline, IL-6 −174 and MT1A +647 polymorphism
Immune functions[69]	NK lytic activity	↑↑	–
	Basal IFN-γ, IL-8, IL- 1ra and IL-6 production	↓	–
	Basal IL-10 and TNF-α production	↓	–
	Stimulated IFN-γ, IL-6, TNF-α, IL-1ra and IL-10 production	↑	–
Jak/Stat signalling and immunomodulation[48,69]	IL-2 and IL-6 STAT3 and STAT5 activation	–	Age of donors, basal zinc status
	Activation-induced cell death	↑	Age of donors, basal zinc status
	Cytokines and metabolic gene expression response to zinc	↑↓	Age, gender and IL-6 −174 and MT1A +647 polymorphism
T cells subsets[69]	Activated T cells (CD3+CD25+)	↓	–
	CD4:CD8	–	–
	Frequencies of CMV-specific cells	–	–

MT, metallothionein; ATF2, activating transcription factor 2; CSF2, colony stimulating factor 2; ICAMI, inter-cellular adhesion molecule 1; LTA, lymphotoxin-α; CCL2, chemokine (C-C motif) ligand 2; SELE, E-selectin; VCAM1, vascular cell adhesion molecule 1; MsR, methionine sulfoxide reductase; SOD, superoxide dismutase; dRib, 2-deoxy-D-ribose; MIP-1α, macrophage inflammatory protein 1α; MCP-1, monocyte chemotactic protein-1; RANTES, regulated upon activation normal T-cell expressed and secreted; CMV, cytomegalovirus; ↑↑ = strongly increased, ↑ = increased, – = not modified, –↑ = slightly increased at least in some sub-groups, ↑↓ = great inter-individual variability.

and its receptor[70]. A comprehensive portrait of the effect of zinc supplementation on zinc status, immune response, cytokines, chemokines and stress-related proteins in old people selected on the basis of IL-6 and MT polymorphisms is provided in Table 2.

Conclusions and future remarks

Even if some controversial findings exist on the 'real' necessity of zinc supplementation, the data reported clearly suggest that zinc plays a pivotal role for the immune efficiency required to reach healthy ageing and longevity. However, the major problem for zinc supplementation in old people is related to the choice of old subjects who effectively need zinc supplementation. The sole determination of plasma zinc is not sufficient, because zinc is bound to many proteins. The intracellular zinc ion availability and the zinc release by MT can be used as complementary methods to test the zinc status, as shown in non-agenarians[16]. The polymorphisms of IL-6 and MT1A may be the added value to screen effective old subjects for zinc supplementation in restoring inflammatory/immune response. As a consequence, healthy ageing and longevity may be achieved. The prolonged survival observed in old, nude and neonatal thymectomized zinc-treated mice and the avoidance of infection relapses in old infected patients after zinc supplementation may be in line with this interpretation. However, some points require further investigation. Firstly, the reason of the limited zinc release in ageing and the biochemical mechanism involved, in particular, addressing NO-related intracellular pathways. However, IL-6 and MT1A polymorphisms may form a solid rationale to select old individuals who effectively need zinc supplementation and not the entire old population.

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