Role of Zinc and Selenium in Oxidative Stress and Immunosenescence: Implications for Healthy Ageing and Longevity

Eugenio Mocchegiani and Marco Malavolta

Contents

Abstract: Ageing is an inevitable biological process with gradual and spontaneous biochemical and physiological changes and increased susceptibility to diseases. Some nutritional factors (zinc and selenium) may remodel these changes leading to a possible escaping of diseases with subsequent healthy ageing, because they are especially involved in improving immune functions as well as antioxidant defense. Experiments performed "in vitro" (human lymphocytes exposed to endotoxins) and "in vivo" (old mice or young mice fed with low zinc dietary intake) show that zinc is important for immune response both innate and adoptive. Selenium provokes zinc release by Metallothioneins (MT), via reduction of glutathione peroxidase. This fact is crucial in ageing because high MT may be unable to release zinc with subsequent low intracellular free zinc ion availability for immune response. Taking into account the existence of zinc transporters (ZnT and ZIP family) for cellular zinc efflux and influx, respectively, the association between ZnT and MT is important in maintaining satisfactory intracellular zinc homeostasis in ageing. Improved immune

E. Mocchegiani (✉) · M. Malavolta
Immunology Ctr. section Nutrigenomic and Immunosenescence
Res Dept. INRCA
Via Birarelli 8
60121, Ancona, Italy
Tel.: +39-071-8004216; Fax: +39-071-206791
E-mail: e.mocchegiani@inrca.it

performance occur in elderly after physiological zinc supplementation, which also induces prolonged survival in old, nude and neonatal thymectomized mice. The association "zinc plus selenium" improves humoral immunity in old subjects after influenza vaccination. Therefore, zinc and selenium are relevant for immunosenescence in order to achieve healthy ageing and longevity.

1 Introduction

Ageing is an inevitable biological process that is accompanied with gradual and spontaneous biochemical and physiological changes including increased susceptibility to diseases, adverse environmental conditions and loss of mobility and agility. Alterations in the immune functions play a fundamental role in ageing. The inability of an organism in remodeling these immune changes may lead to the appearance of some degenerative age-related diseases. As a result, the "remodeling theory of ageing" has been proposed (Paolisso et al. 2000). Various nutritional factors are directly linked with these phenomena as for instance in restoring the immune functions as well as in the capacity to respond to oxidative stress (Meydani 2001), which is in turn the main cause of the immune derangement in elderly (Pawelec 2000).

Approximately, 40 micronutrients (vitamins, essential minerals and other compounds required in small amount for normal metabolism) have been reported as essential components in the diet (Shenkin 2006). The dietary intake of essential macro and micronutrients is usually inadequate in the elderly (Ames 2006). Several causes contribute to this gap. First of all, the poor socio-economic condition present in a large part of old people may lead to a consumption of inexpensive foods deficient in micronutrients, such as carbohydrates (Kant 2000). The gap is worsened by loss of appetite, lack of teeth, intestinal malabsorption and decreased requirement of energy that lead to the final result of frailty, disability and mortality (Semba et al. 2006). Some authors have reported that the deficiency of macro and micronutrients in ageing is strictly related to global impairments of the immune functions with subsequent limited defense against external noxae and appearance of age-related diseases (Lesourd 2006). By contrast, recent longitudinal studies in dietary daily intake in human nonagenarian/centenarians (successful ageing) have shown that an adequate consumption of micro and macronutrients as well as a satisfactory content of some trace elements in the cells lead to good performances in several immune functions, especially in innate immune performances (Chernoff 2001; Mocchegiani et al. 2003). Therefore, nutritional factors may play a pivotal role for immunosenescence in order to reach healthy ageing and longevity. We herein review the role of zinc and selenium, taking into account the pivotal role played by these two micronutrients in the efficiency of the immune functions (Buttriss 2000). Recent epidemiological and clinical evidence have shown that in most developing countries deficiencies of these micronutrients are partly responsible for the severity of infectious disease, morbidity and mortality in malnourished children (Bhaskaram 2002) as well as in ageing (Meydani 2001). Indeed, these two trace elements form an important pillar in the nutrition

of young and elderly persons because also involved in tissue integrity (Enwonwu and Sanders 2001). Thus, it is evident that a deficiency of these elements could lead to a crucial impairment of the organ and tissue function with subsequent influence on many body homeostatic mechanisms, including the immune functions.

2 Zinc

2.1 Zinc Biology

Zinc is one of the most important trace elements in the body, although its presence in nature does not exceed 0.02% (Mills 1989). The major characteristics of zinc include a highly concentrated charge, a small radius (0.65A), no variable valence [low risk of free radical production], ready passage from one symmetry in its surroundings to another without exchange, rapid exchange of ligands (on and off reactions), and binding mostly to S- and N-donors in biological systems. These properties enable zinc to play a major biological role as a catalyst. Removal of the catalytic zinc results in an active apoenzyme that usually retains the native tertiary structure (Vallee and Falchuk 1993). Thus, it is not surprising that zinc is essential for the activity of more than 300 enzymes influencing the activity of zinc dependent antioxidant enzymes, such as superoxide dismutase (SOD) and various organ functions having a secondary effect on the immune system (Rink and Gabriel 2000).

Zinc also regulates the balance between the gene expression of metalloproteinases (MMPs) and the tissue inhibitors of matrix metalloproteinases (TIMPs; Nagase and Woessner 1999). The main function of MMPs is the removal of extracellular matrix (ECM) during tissue resorption and progression of many diseases. However, it is notable that MMPs also alter biological function of ECM macromolecules by specific proteolysis (Shapiro 1998). Therefore, since MMPs are induced especially by proinflammatory cytokines (IL-1 and TNF-alpha), an overexpression of MMPs may lead to excessive proteolysis of ECM, as it occurs in chronic inflammation (Gueders et al. 2006). As a consequence, degradation of ECM and limited cell–cell adhesion may occur, so "trapping" bioactive mediators (Moot and Werb 2004). Thus, the expression of MMPs genes are under the control of some inhibitors of MMPs, such as TIMPs gene products, α-2 macroglobulin (α-2M) and 13-amyloid precursor protein (Nagase and Woessner 1999). As a result, a balance in the expression of the metalloproteinases [either as activators (MMPs) or as inhibitors (TIMPs)] is necessary for an optimal function of many biological systems. Examples of altering the balance between MMPs and TIMPs or α-2M have been recorded in certain types of cancer, infections and ageing (Nagase and Woessner 1999) that are conditions characterized by zinc deficiency (Fabris and Mocchegiani 1995). Zinc also regulates G0/G1 phase of cell cycle through Cyclins/CDK complexes in a dose dependent manner. Specifically, high doses of zinc (900 μM) result in cell cycle arrest (Paramanantham et al. 1996), whereas low doses of zinc (150 μM) inhibit apoptosis (Fraker 2005). Zinc is present in "zinc finger domains" of many proteins, peptides,

enzymes, hormones, transcriptional factors and cytokines, which act in maintaining body homeostasis (Coleman 1992; Berg and Shi 1996). Zinc also regulates mRNA stability (Taylor and Blackshear 1995) and extracellular matrix (Vallee and Falchuk 1993). Moreover, zinc binds enzymes, proteins and peptides with different binding affinity (kd) ranging from 10^{-2} to 10^{-14} mol/L (*See* review Mocchegiani et al. 1998). These compounds display low biological activity when the zinc-binding doesn't occur, as for instance for thymic hormone named thymulin, which loses its activity in absence of zinc (Fabris et al. 1984). Finally, zinc plays a critical role in structure, function, stabilization and fluidity of biomembrane due to its binding to sulphydryl groups forming mercaptides (Vallee and Falchuk 1993).

Zinc also maintains the enzymatic activity of inducible nitric-oxide synthase (iNOS; Bodgan et al. 2000), with a binding between zinc and two cysteine residues, which are part of the structures of the heme domain of iNOS (Li et al. 1999). As: (i) Nitric Oxide (NO), via NO synthases, affects the gene expression of metallothioneins (MT) in order to protect the host from oxidative stress (Arizono et al. 1995) and (ii) NO is involved in zinc release from MT, via s-nitrosylation (Zangger et al. 2001), the structural task of zinc in NO production is crucial.

In this context, the release of zinc by MT, via s-nytrosilation, contributing to raise the intracellular free zinc ions concentration, plays a crucial role in modulating the production of proinflammatory cytokines and in the activation of immune cells (Rink and Haase 2007). Therefore, the interrelationships between zinc and MT is crucial in maintaining the immune response especially in ageing where the production of proinflammatory cytokines is chronic leading to a constant presence of inflammatory status coupled with low intracellular zinc ion bioavailability (Mocchegiani et al. 2004). The interrelationship between zinc and MT is also regulated by the special proteins named zinc transporters (ZnT), which in turn appear to be also specifically involved through regulation of cellular zinc homeostasis via influx, efflux, or vesicular sequestration (Cousins and McMahon 2000; Eide 2006). The ZnT, some of which are tissue specific, maintain intracellular zinc concentration in a narrow physiological range in order to avoid cellular zinc toxicity or deficiency when dietary zinc intakes fluctuate. Two families of ZnT have been identified. The ZnT family decreases cytoplasmic zinc concentrations by secretion, sequestration, or efflux, whereas the ZIP family increases cytoplasmic zinc influx or release of stored zinc (Eide 2006). Therefore, the balance of ZnT is fundamental to maintain an optimal intracellular zinc homeostasis in ageing, because reduced zinc intake by the diet or intestinal zinc malabsorption or loss of zinc through urine by high levels of proinflammatory cytokines are usual events in elderly (Prasad et al. 1993b).

2.2   *Zinc-Metallothioneins and Ageing*

MT, are a group of low-molecular-weight metal-binding proteins who have high affinity for zinc (kd = 1.4×10^{-13} M; Kagi and Schaffer 1998). MT exist in different isoforms characterized by the length of aminoacid chain: isoform I, II, III e IV mapped on chro-

mosome 16 in man and on chromosome 8 in mice with complex polymorphisms (West et al. 1990). The more common isoforms are I and II; the isoform III, also called growth inhibitory factor (GIF), is a brain-specific member of the MT family and the isoform IV is restricted in 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 (Maret and Vallee 1998). The zinc/cysteine clusters are of two different types. In the beta-domain cluster, three bridging and six terminal cysteine thiolates provide a coordination environment that is identical for each of the three zinc atoms. In the alpha-domain clusters, there are two different zinc sites; two of them have one terminal ligand and three bridging ligands respectively, while the other two have two terminal and two bridging ligands (Maret and Vallee 1998).

Following these biochemical characteristics, MT distribute intracellular zinc as zinc undergoes rapid inter- and intracluster exchange (Kagi and Schaffer 1998). Moreover, MT act as antioxidant since zinc-sulfur cluster is sensitive to changes of cellular redox state and oxidizing sites in MT (reduced thiol groups) induce the transfer of zinc from its MT binding sites to those of lower affinity in other proteins (Kagi and Schaffer 1998). This transfer confers biological activity to antioxidant metalloenzymes. Therefore, the redox properties of MT and their effect on zinc in the clusters are crucial for the protective role of MT in presence of ionizing and UV radiations (Cai et al. 1999), heavy metals (mercury, cadmium), lipid peroxidation, reactive oxygen species, oxidative stress caused by anticancer drugs, and conditions of hyperoxia (Sato and Kondoh 2002). This protective role of MT has been studied especially in young-adult MT knockout mice (null mice) for short periods of exposure to toxic metals, such as cadmium for 10 weeks (Habeebu et al. 2000) or mercury (one single injection and the effect of mercury analyzed 3 days after the injection; Satoh et al. 1997), or to anticancer agents for 48–72 hrs. (Kondo et al. 1997) or in presence of an excess of zinc or zinc deficiency for 3 weeks (Kelly et al. 1996). Therefore, the protective role of MT is evident in transient stress condition, as it may occur in young adult-age, in which the chronic status (by stress or inflammation) is a rare event (Mocchegiani et al. 2006). In contrast, this role may be questionable in ageing because the stress-like condition and inflammation by high levels of IL-6 are chronic (Ashok and Ali 1999), with also a different response to stress with respect to young (DeGroot et al. 2006). Since IL-6 affects the gene expression of MT (Hernandez et al.2000), these proteins may turn off from protective to harmful agents in ageing following the "antagonistic pleiotropy theory of ageing" (Williams and Day 2003). In fact, despite MT increase in ageing, a limited release of zinc by MT leading to an impaired immune and antioxidant response has been proposed (Mocchegiani et al. 2000a, b). In contrast, in presence of lower stress and inflammation, as it occurs in centenarians, MT production is low coupled with satisfactory zinc ion bioavailability (Mocchegiani et al. 2002a). Indeed, since IL-6 acts on the cells through its subunit receptor gp130 (Bravo and Heath 2000), the relative lower gene expression of gp130 with respect to elderly found in centenarians (Moroni et al. 2005) may imply that a quota of IL-6 is inactive in centenarians leading to low gene expression of MT, satisfactory free zinc ion availability and low degree of inflammation (Mocchegiani et al. 2002a). As a result, the satisfactory immune performances and antioxidant activi-

ties lead to a good healthy status in these exceptional individuals (Mecocci et al. 2000; Mocchegiani et al. 2002a). Therefore, the interrelationships among inflammatory status, MT and zinc are pivotal in order to achieve successful ageing, furtherly suggesting a different role of MT in ageing that is crucial for immune response (Mocchegiani et al. 2000a). Whether MT might play an antagonistic pleiotropic role remains however to be clearly demonstrated also taking into account that they may play different role in different organs. On this aspect, recent findings in cardiac-specific Metallothionein transgenic mice suggest that the expression of these proteins in cardiocytes may alleviate aging-induced cardiac contractile defects and oxidative stress prolonging life span (Yang et al. 2006). In addition, Daf-2 mutant nematodes other than a longevity phenotype, display an altered expression of MT which, in turn, seems to interact with the insulin signaling pathway (Barsyte et al. 2001). Therefore, even if the specific function of MT in ageing is still a matter of discussion, all these reports associated to recent findings on the possible role played by MT in modulating cellular respiration and energy metabolism (Feng et al. 2005; Ye et al. 2001) strongly suggest that these proteins are involved in the maintenance of health status and in successful aging. On the other hand, recent findings show a novel polymorphisms of MT1A (A/C at position +647 leading to an asparagine/threonine aminoacid substitution) involved in successful ageing, lower inflammation and satisfactory intracellular zinc ion bioavailability (Cipriano et al. 2006).

2.3   Zinc Transporter and Ageing

With regard to the role played by the ZnT in ageing and immunosenescence, a paucity of data exists in literature. After an increase from the birth up to adult age in some tissues, pancreas (Clifford and MacDonald 2000) or brain (Nitzan et al. 2002), significant decrements of both ZnT and ZIP families in peripheral leukocytes from elderly women occur, in particular the subtypes ZnT1 and ZIP1 (Andree et al. 2004). Taking into account that ZIP family increases cytoplasmic zinc influx (Eide 2006), an intriguing point is that Zip14 expression is up-regulated through IL-6, and that this zinc transporter most likely plays a major role in the mechanism responsible for an excess of intracellular zinc and, at the same time, for hypozincemia that accompanies the acute-phase response to inflammation and infection (Liuzzi et al. 2005). Since chronic inflammation by high IL-6, hypozincemia and risk of infections are usual events in old age (Mocchegiani et al. 2003), the possible alterations of the ZnT in ageing coupled with the inability of high zinc-bound MT in zinc release, may thus allow still more synergistic deleterious effects on immune response that it may be due or to low or excess of zinc within the cells. This last assumption is supported by the discovery that both low and high levels of intracellular zinc lead to cell death (Fraker 2005). Therefore, the intracellular zinc ion availability should be maintained within a strict range in order to exert beneficial effect, otherwise it may trigger pathological pathway cascades possibly contributing to the onset and progression of degenerative diseases (Mocchegiani et al. 2006).

2.4 Zinc-MT and Immunosenescence

For a prompt immune response against stressor agents and inflammation, macrophages produce some cytokines, such as IL-1, IL-6, 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 (Bui et al. 1994). These findings clearly suggest the existence of interplay between MT and the immune system. IL-1 affects MT mRNA in thymic epithelial cells (TECs) by means of PKC, which is, in turn, zinc-dependent (Coto et al. 1992) and participates in metal-induced MTmRNA (Yu et al. 1997). Moreover, MT are donors of zinc for thymulin reactivation in TECs (Coto et al. 1992). MT act both as a reservoir of zinc during zinc deficiency and as a zinc buffering protein in presence of excessive amount of zinc in order to prevent zinc toxicity (Kelly et al. 1996). Following these findings, MT are, out of doubt, protective agents with also the task in preventing zinc deficiency during an inflammatory status. It has been recently reported that, under inflammatory conditions, MT in the extracellular environment may support the beneficial movement of leukocytes 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 responses that regulate leukocyte trafficking (Yin et al. 2005). Taking into account that zinc ions attract leukocytes by inducing and promoting the chemotactic response (Hujanen et al. 1995), high MT production might be dangerous for immune response in presence of chronic inflammation. Moreover, (i) the existence of high MT and low zinc ion bioavailability in the atrophic thymus from old mice (Mocchegiani et al. 2004); (ii) the presence of high MT in lymphocytes from old people and Down's syndrome subjects (syndrome of accelerated ageing) coupled with impaired innate immunity (Mocchegiani et al. 2002a) and (iii) the occurrence of atrophic thymus in young stressed mice overexpressing MT (Mocchegiani et al. 2002b), furtherly suggest this dangerous role played by MT in immune function during ageing. Additionally, elevated levels of extracellular MT, as it can be found especially in chronic inflammatory sites, can cause a dramatic decreases in cytotoxic T lymphocyte (CTL) activity against allogeneic target cells, reduces the proliferative response of CTLL-2 cells to cytokines, and decreases the level of major histocompatibility complex (MHC) Class I and CD8 molecules detectable on the surface of lymphocytes (Youn and Lynes 1999). Therefore, high MT may also have an immunosuppressive effect worsened by the fact they are not donors of zinc in ageing but rather sequester zinc. 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 (Kagi and Schaffer 1998). An unbalance between MT isoforms leads to impairments of zinc-dependent body homeostatic mechanisms within the brain, as reported in SAMP10 mice (model of accelerated ageing; Wen et al. 2006). Moreover, high MT are an index of unfavorable prognosis in cancer (Ebadi and Swanson 1988).

However, the limited capability of MT in zinc release is still unresolved problem in ageing, especially regarding to the precise mechanism involved. The zinc release

from MT under oxidative stress conditions is accompanied by more MT disulfide bond formation (Feng et al. 2006). But, an intriguing point is that also NO provokes the zinc release by MT, via s-nitrosylation (Zangger et al. 2001). Despite iNOS increases in ageing, the release of zinc by MT is very limited. One hypothesis might be an unbalance between NO synthases (iNOS and cNOS; Mocchegiani et al. 2000a). However, 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 for testing intracellular free zinc ion availability in PBMC recently developed in our laboratory (Malavolta et al. 2006), it has been shown that the NO-induced release of zinc can be preserved at least in nonagenarians carrying MT1A polymorphism favorable for successful ageing (Cipriano et al. 2006). Moreover, a flow cytometric assay for the measurement of intracellular labile zinc was recently developed by Haase et al. (2006) The zinc-sensitive fluorescent probe named FluoZin-3 was used to quantify the amount of labile zinc in peripheral blood mononuclear cells isolated from human blood. With this method, the intracellular concentrations of labile zinc in resting cells were estimated to be 0.17 nM in monocytes and 0.35 nM in lymphocytes (CD4+; Haase et al. 2006). Therefore, 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 intracellular labile zinc. Anyway, a limited zinc release from MT exists in ageing provoking a low free zinc ion availability for immune response and antioxidant activity. The recent discovery of another novel polymorphism of MT (-209A/G MT2A) may indirectly support this assumption. Indeed, old subjects carrying AA genotype display high MT, low zinc ion availability, enhanced IL-6 and impaired innate immune response with subsequent possible risk for atherosclerosis and diabetes type II (Giacconi et al. 2005). Therefore, MT may have a different role in immunosenescence, following the concept that several genes/proteins that increase fitness early in life may also have negative effects later in life: named "Antagonistic Pleiotropy Theory of Ageing"(Williams and Day 2003).

2.5 Rationale for Zinc Supplementation in Ageing: "In Vitro" Studies

Since the crude zinc balance is negative in old mice (Mocchegiani et al. 1995) and in old human (Turnlund et al. 1986), zinc supplementations in old mice and in elderly have been carried out in order to improve the immune response. The scientific rationale for the immune supporting role of zinc supplementation "in vivo" finds consistent support by data obtained "in vitro" in immune cells.

At this regard, many effects of zinc on immune cells have been shown by assessing the cytokine concentration in the samples after zinc stimulation. When PBMCs are stimulated with zinc, IL-1, IL-6, TNF-α, soluble (s)IL-2 receptor and IFN-γ are released (Ibs and Rink 2003). The secretion of IL-1, IL-6 and TNF-α is induced directly by zinc in monocytes and is independent by the presence of

lymphocytes (Driessen et al. 1994). However, the effect of zinc on monocytes may depend upon external stimulation. In fact, zinc inhibits LPS-induced TNF-α and IL-1β release from primary human monocytes and monocytic cell lines through the inhibition of cyclic nucleotide phosphodiesterase activity (von Bulow et al. 2005), suggesting that zinc may display also some anti-inflammatory properties.

The dose of zinc used is also a critical variable. In serum-free culture medium, concentrations >100 μM of zinc/L stimulate monocytes but prevent T-cells from activating, perhaps due to the lower intracellular content in T-cells than in monocytes (Ibs and Rink 2003).

Treatment with zinc "in vitro" generally displays also 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 (Clegg et al. 2005; Wiseman et al. 2006). Therefore, the modulation of the zinc homeostasis plays a key role not only in preventing apoptosis, when oxidative stress is low, but also in inducing apoptosis, when oxidative stress and cellular damage is high, in order to down regulate immune responses and to eliminate virally infected or malignant cells (Fraker and Lill-Elghanian 2004). Taking into account the strict correlation existing between oxidative stress and immune function especially in response to specific stimuli through the production of proinflammatory cytokines for a prompt immune response (Franceschi et al. 2005), this role of zinc in inducing apoptosis of only damaged cells in presence of high oxidative stress is evident in young-adult age and with a great surprising in very old age (Ostan et al. 2006), perhaps due to the presence of satisfactory zinc ion availability (Mocchegiani et al. 2002a) that regulates p53 activity for health lifespan (Bauer and Helfand 2006), being p53 a zinc binding protein (Hainaut and Mann 2001).

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 (Provinciali etal. 1998), whereas the direct introduction of free zinc as zinc-pyrithone inside thymocytes induces apoptosis (Mann and Fraker 2005). In this last case, the continuous presence of intracellular free zinc ions can advice the cell that permanent oxidative stress and irreversible damage are present, thus activating proapoptotic pathways.

2.6 Effect of Zinc Supplementation in Ageing

2.6.1 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, such as the decreased circulating thymic hormone levels (Iwata et al. 1979). More recently, a physiological zinc supplementation (18 μg/ml Zn^{++} in the

drinking water for 1-month) in old mice induces thymus re-growth and functionality (Dardenne et al. 1993; Mocchegiani et al. 1995) and restoration of NK cell cytotoxicity (Mocchegiani et al. 1995). That the benefit of zinc supplementation upon the immune functions in old mice is not to consider an epiphenomenon comes by the analysis of the rate of survival in old zinc treated mice. Old mice (inbreed Balb/c mice) treated with daily zinc at the dose reported above in drinking water from the pre-senescent age (12–14 months of age) display a significant increment of the rate of survival up to 33th month of age when this strain of mice usually lives up to 28–29th month of age. The increment of old survivor zinc treated mice is particularly significant in the middle age (24–25th month of age; Mocchegiani et al. 2000b). The increased rate of survival is largely due to significant decrements of deaths due to cancer and infection in the middle age (Mocchegiani et al. 2000b). Of interest, the crude zinc balance is negative, other than in old mice, also in nude and neonatal thymectomized mice (Mocchegiani et al. 1995 2000b 2007). A zinc supplementation increases the rate of survival also in nude and neonatal thymectomized mice (Mocchegiani et al. 2007), which display a very short survival due to thymus absence (Piantanelli and Fabris 1978). Taking into account that the liver extrathymic T-cell pathway is prominent in nude, thymectomized and old mice in order to compensate the thymic failure (Abo 2000), it is evident the zinc also affects the liver extrathymic T-cell pathway with good performances of the immune functions against external noxae (Mocchegiani et al. 1998) coupled with increased rate of survival.

2.6.2   Elderly

With regard to elderly, undefined data exist on the beneficial effect of zinc supplementation upon the immune efficiency due to different doses of zinc used and to the length of the treatment (Bodgen et al. 1990; Boukaiba et al. 1993; Cakman et al. 1997; Duchateau et al. 1981; Fortes et al. 1998; Prasad et al. 1993b; Sandstead et al. 1982). Although zinc was used at the dose recommended by RDA (from 15 to 25 mg/day) in the majority of the studies, Prasad et al. (1993b) and Boukaniba et al. (1993) have found an increment of thymulin activity and improvements in response to skin-test antigens and taste acuity (zinc dose = 15 mg /day for 4 months); Bodgen et al. (1990) have reported no benefit exclusively for increased lymphocyte mitogen proliferative response (zinc dose = 15 mg/day for 1-year); Cakman et al. (1997) have found enhanced IFN-γ production by leukocytes (zinc dose = 15 mg/day for 45 days); Fortes et al. (1998) report an increased number of CTLs (zinc dose = 25 mg/day for 40 days); Duchateau et al. (1981) and Sandstaed et al. (1982) have observed an improvement in response to skin-test antigens and taste acuity (zinc dose = 220 mg/day for 1-month). Thus, it seems evident from these studies that physiological dose of zinc for a long period or high doses of zinc for short periods might induce limited effects on immune response perhaps due to a zinc accumulation in various organs and tissues with subsequent toxic effect of zinc upon the immune functions (Fosmire 1990; Sandstead 1995). In this context, it is useful to remind that high

doses of zinc trigger apoptosis of the immune cells in presence of high oxidative stress, as reported above. Therefore, zinc supplementation has to be used with caution for short periods and on alternate cycles. Following that, in our experience, zinc treatment at the dose of 15 mg Zn^{++}/day for 1-month in Down's syndrome subjects, in elderly and in old infected patients restores thymic endocrine activity, lymphocyte mitogen proliferative response, CD4+ cell number, peripheral immune efficiency (NK cell cytotoxicity), Th1/Th2 paradigm (Franceschi et al. 1988; Kahmann et al. 2006; Mocchegiani et al. 2003) and DNA-repair (Chiricolo et al.1993). At clinical level, significant reductions of infection relapses occur in Down's syndrome (Licastro et al. 1994) in elderly and in old infected patients with a faster outcome from the pathology (Mocchegiani et al. 2003).

Physiological zinc supplementation was reported to lead to a decrement in plasma lipid peroxide concentrations in elderly people living in a public home (Fortes et al. 1997). The positive effect of zinc on lipid peroxide could derive from its protective effects on sulphydryl groups against oxidation and the fact that zinc is a component of superoxide dismutase (SOD; Mills 1989).

Zinc supplementation is also useful in reducing the oxidative stress in old patients with diabetes type II (Roussel et al. 2003) because it inhibits NF-kB activation and decreases inducible NO synthase. As such, the generation of ROS decreases, thus zinc provides a protective effect on β cells against death (Ho et al. 2001).

An intriguing point of the zinc supplementation is the increment of ZnT. Elderly women treated for 27 days with 22mg of zinc gluconate /day display significant increments of ZnT1 gene expression in peripheral leukocytes (Andree, et al. 2004), even if the gene expression of the ZnT is sensitive in relation to the immune cells considered (Whitney et al. 2003). Such increments of ZnT1 have been also observed in human lymphoblastoid cells adding in vitro 50 or 100 μmol/L of zinc (Andree et al. 2004), furtherly suggesting the relevance of zinc supplementation also in affecting the gene expression of ZnT and, consequently, the correct maintenance of intracellular zinc homeostasis.

That the beneficial effects of zinc supplementation are not to be considered as epiphenomena, it comes by the increased survival also in nude and neonatal thymectomized (nTx) mice treated with physiological zinc (18 μg Zn++/day for 1-month) in the drinking water, taking into account that they display a very short survival due to thymic absence and negative crude zinc balance (Mocchegiani et al. 1995, 2002b, 2007). The prolonged survival is largely due to mortality reduction (about 50%) by infections because zinc also affects the extrathymic T-cell pathway that is prominent in old, nude and nTx mice for T-cell maturation and host defense (Abo et al. 2000). Indeed, in vivo and in vitro studies have shown that zinc is a key trace element for liver T-cell maturation and function, particularly for liver NKT cells bearing TCR γδ with high production of IFN-γ (Mocchegiani et al. 2004). Of interest, the increment and function of NKT cells (Miyaji et al. 2000) and Tγδ cells (Colonna-Romano et al. 2002) also occur in human centenarians, who in turn display satisfactory zinc ion bioavailability and good immune response (Mocchegiani et al. 2002a).

All these "in vitro" and "in vivo" studies in ageing, some age-related diseases, and syndrome of accelerated ageing (nude mice, nTx mice, Down's Syndrome)

demonstrate the pivotal role played by zinc supplementation in maintaining or improving global immune response and in fighting the oxidative stress, strengthen by findings observed in human centenarians.

However, since zinc also affects MT gene expression (Maret 2003), the question arises whether zinc supplementation in old age may furtherly increase MT causing possible major harmful effects. Old zinc treated mice exhibit no further significant increments of liver MT mRNA, suggesting that MT in ageing may be already over-expressed before supplementation (Mocchegiani et al. 2002b). Moreover, the effects observed during zinc supplementation on the immune system, such as reduced inflammation and restored Th1/Th2 paradigm (Prasad 2000), suggest that intracellular zinc may return available despite over-expressed MT (Mocchegiani et al. 2002b) with a maintenance of their original protective role. Therefore, the possible harmful effect of MT in ageing seems to not constitute a problem during physiological zinc supplementation.

2.7   Zinc Interaction with Other Micronutrients and Zinc Toxicity

The beneficial effect of physiological zinc supplementation must be, however, related to the levels of other cations such as cadmium, lead, calcium, iron, manganese and copper. The beneficial effects of zinc on ameliorating toxicity of cadmium and lead, accentuation of zinc deficiency by administration of calcium and phytate, and production of hypocupremia by excessive zinc intake in humans and animals, are some examples of competition phenomena between these cations (Hill 1976). Such a competition occurs because these ions have similar valence shell electronic structure and, therefore could be antagonist to each other. For instance, the competition between zinc and iron (Fe++) occurs at the level of cysteine-histidine ligands for the formation of iron or zinc "fingers" proteins (Prasad 1993a). If iron is excess, a preferential binding of iron than zinc to the metal free-protein occurs. Excess of zinc or zinc deficiency impairs DNA-protein interactions of zinc-fingers domains with their cognate DNA target sites. In these conditions the production of some transcriptional factors like SP1 or TFIIIA is impaired (Thiesen and Bach 1991). The same impairment of zinc fingers DNA domains occurs in excess or deficiency of copper (Prasad 1993a). This reinforces the notion of the relevance of interactions between zinc and copper as well as with other metals in the immune efficiency (Sandstead 1995). Thus a limited range of bioavailability exists for each metal. As such, immune responses are optimum. Indeed, the beneficial effect of zinc is strictly dependent by the dose and the length of treatment. Zinc accumulation or imbalance zinc-to-copper ratio may occur despite low doses of zinc (Fosmire 1990). As such, harmful side effects in the cardiovascular system and in the brain may appear with increased low-density lipoprotein and cholesterol (Fosmire 1990) and neural cell-death (Kim et al. 1999), respectively. Therefore, caution in zinc supplementation is necessary for avoiding undesirable and harmful unexpected side effects. Zinc supplementation must not exceed 2–3 times the RDA/day, for short periods

(1–2 months) and on alternate cycles. This treatment doesn't interfere in copper absorption (Faillet-Coudray et al. 2006; Licastro et al. 1994). Zinc picolinate form may be the best supplement (Wapnir et al. 1983).

3   Selenium

3.1   *Selenium Biology*

Selenium (Se) is an essential dietary element for the prevention of some diseases, including cancer and infections (Schwarz 1976). Such an assumption has been subsequently confirmed in animals with a selenium deficiency in the diet and concomitant treatment with various carcinogens, such as 1,2-dimethylhydrazine (DMH) or dimethylbenz(a)anthracene (DMBA), compared with animals fed with higher content of selenium in the diet. In this context, although Se deficiency appears to affect DMH toxicity with however no inhibition of tumor development by nutritional Se (0.1 ppm Se; Pence and Buddingh 1985), three relevant papers report a greater development of carcinoma by DHM or DMBA in various organs (colon and mammary gland) in rats fed with selenium deficiency in the diet in comparison with rats treated with 5 ppm of Se (Jacobs 1983; Liu and Milner 1992; McGarrity and Peiffer 1993). These findings further suggest the ability of dietary selenium to inhibit the in vivo metabolism of carcinogens DMBA or DMH with subsequent less development of the tumor. With regard to infection, decreased dietary selenium can change a normally avirulent B3 coxsackievirus (CBV3/0) into a virulent virus (CBV3/20) by inducing changes in viral genoma, especially in viral RNA polymerase mutations (Duarte et al. 1994) that infect heart muscle and cause myocarditis with subsequent possible development of dilated cardiomyopathy and death (Beck and Levander 2000). In food, selenium derives from vegetables and animal products and in particular from the consumption of seafood, liver, and cereals. However, in vegetables and cereals the amount of selenium varies in soil in different countries and geographical regions (Wasowicz et al. 2003). Indeed, selenium deficiency and related diseases have been well documented in geographic regions where the soil content is low, such as the Chinese province of Keshan (Li et al. 1985). From this Region of China, in fact, Keshan disease is named the pathology characterized by selenium deficiency and presence of substantial number of virulent viruses, including coxsackieviruses (Li et al. 1995).

Mammals can use both inorganic and organic selenium as a nutrient. Most of the biological functions of selenium are attributed to selenoproteins, which contain selenocysteine residues responsible for their specific activity. Selenoproteins are present in every cell type. The human selenoproteome consists of 25 selenoproteins, mostly involved in antioxidant defence systems (Kryukov et al. 2003).

Glutathione peroxidases (GPxs), a family of the selenoproteins, protect cells against oxidative damage by catalysing the reduction of hydrogen peroxide and other hydroperoxides (Brigelius-Flohe 1999; Hall et al. 1998). Five selenium dependent

GPx isoforms exist in humans and four isoforms in mice. GPx1 is found in the cytosol of almost all cells and catalyses the reduction of free hydroperoxides. GPx2 is expressed in the gastro-intestinal tract and has a substrate specificity similar to GPx1; GPx3 is an extracellular enzyme found in plasma and reduces membrane-bound phospholipid hydroperoxides (Brigelius-Flohe 1999). GPx4 is expressed in various tissues, and reduces phospholipid hydroperoxide and hydrogen peroxide using also thiols, such as 2-mercaptoethanol, cysteine and homocysteine, other than GSH as reductant agents (Roveri et al. 1994). The isoform GPx6 seems to be specifically expressed in embryonic tissues and olfactory epithelium (Kryukov et al. 2003). It also exist a selenium independent isoform, GPx5, which is an epididymis isoenzyme present in mice and humans (Hall et al. 1998), but its mRNA was found to be not translated into functional protein in human epididymis (Ghyselinck et al. 1993). Selenium is also involved in the thioredoxin system, a major enzymatic system that plays an important role in maintaining the redox state of the cell (Holmgren 1985). This system is highly complementary to the GSH system in protecting against oxidative stress (Watson et al. 2004). It comprises basically of thioredoxin (Trx) and the selenoprotein thioredoxin reductase (TR) and uses the reducing power of NADPH to act as a potent antioxidant system as well as a general disulfide redox system (Rundolf et al. 2004). Mammalian TR maintains Trx in a reduced state (Holmgren 1985) and reduces a variety of other substrates including nondisulphides. The thioredoxin system protects the cell against oxidative stress through a variety of mechanisms. Trx can directly quench singlet oxygen and scavange hydroxyl radicals (Das and Das 2000), or reduced Trx can indirectly serves as an electron donor for Trx peroxidase. In addition, human TR is directly capable to efficiently reduce lipid hydroperoxides, hydrogen peroxide and organic hydroperoxides using NADPH, especially in the presence of catalytic amount of selenocysteine, thus serving as an important alternative to the Gpx pathway for the elimination of harmful hydroperoxides (Bjornstedt et al. 1995). Trx system is also critical for signal transduction (Arner and Holmgren 2000) and in the restoration of the reduced form of several antioxidant compounds, including ascorbic acid, lipoic acid, and ubiquinone (Nordberg and Arner 2001). In this context, selenomethionine, a potent catalytic antioxidant in biological system and an aminoacid occurring in proteins in place of methionine (Walter and Roy 1971), reacts more efficiently than methionine (Padmaja et al. 1996) with oxidants forming methionine selenoxide which, in turn, is effectively and rapidly reduced to seleniomethionine by glutathione (Assmann et al. 1998). In contrast, methionine sulphoxide that it is produced by the oxidation of methionine in presence of oxidants, is not simply reduced by GSH, but it requires a specific enzymatic reaction catalyzed by methionine sulphoxide reductase (Levine et al. 1996). Since selenomethionine can occur in proteins such as haemoglobin (Beilstein and Whanger 1986), these residues may play a defensive role against peroxinitite.

Another selenoprotein, which reduces phospholipid hydroperoxides in the presence of thiols, is the Selenoprotein P (SeP; Burk et al. 2003). SeP is expressed in many tissues and represents the major plasma selenoprotein, which contains 50% of the total plasma selenium in the form of selenocysteine. SeP protects endothelial

cells against damage from peroxynitrite and transports selenium from the liver to peripheral tissues (Burk et al. 2003).

Last, but not the least in order of importance, is a class of selenoproteins (iodothyronine deiodinase enzymes), which catalyse the peripheral deiodination of thyroxin (T4) to 3,3'5-triiodothyronine (T3). These enzymes play crucial roles in determining the circulating and intracellular levels of T3 and, consequently, the control of growth, development, differentiation, metabolism and finally also the immune response (Kohrle 2000; Beckett and Arthur 2005).

Immunologically, the ability of selenoproteins to protect the host from oxidative stress is vitally important, since many host defence systems rely on the microbiocidal effects of macrophage- or neutrophil-generated free-radical species. Oxidative species are generated through general metabolism, during the metabolism of xenobiotics and during exposure to ultraviolet radiation (UV) in sunlight. Inflammation as a process to clear infection and damaged tissue also generates great oxidative stress. If antioxidant systems are not functioning correctly, host cells will be damaged (McKenzie et al. 1998). Taking into account that the inflammation is chronic in ageing as well as oxidative stress (Franceschi et al. 2000), the role played by selenium through the selenopreoteins in immune response is therefore vital in elderly.

3.2 Selenium and Immune Function

The influence of selenium on the immune function can be, in part, attributed to the same selenoproteins involved in the protection against oxidative damage and, in part, to still undefined biochemical pathways. The antioxidant GPxs have probably a role in protecting neutrophils from ROS that are produced during inflammation (Arthur et al. 2003a, b). Selenium supplementation, in mice, increases the expression of subunits alpha (p55) and/or beta (p70/75) of IL-2 receptor (IL-2R) from activated lymphocytes and NK cells, thereby enhancing proliferation and clone expansion of cytotoxic precursor cells. In vitro, selenium enhances the release of tumor necrosis factor (TNF), IL-1 and IL-6 from LPS stimulated macrophages (*See* review Beckett et al. 2003). However one of the most widely investigated associations between selenium and the immune system is the effect of the micronutrient on neutrophil function. Neutrophils produce superoxide-derived radicals to take part in killing of microbes. This type of process is a balance between the production of sufficient radicals to kill invading organisms and the systems that protect the neutrophils themselves from the radicals. Thus, although selenium deficiency does not affect neutrophil numbers in a range of species, certain aspects of their function are defective (Turner and Finch 1991). Neutrophils from selenium-deficient mice, rats and cattle are able to ingest pathogens in vitro but are less able to kill them than are neutrophils from selenium-sufficient animals. This defective function has been associated with decreased cytosolic GPx (GPx1) activity in the neutrophils, which allows the free radicals that are produced in the respiratory burst to kill the neutrophils themselves (Arthur et al. 2003b).

Therefore, taking into account all these mechanisms, selenium deficiency has been mainly studied in relation to ageing/mortality and in some age-related diseases, whose pathogenesis is related to preservation of membrane integrity and to oxidative damage of biomolecules, such as lipids, lipoproteins and DNA.

3.3   Selenium, Ageing and Age-related Diseases

Selenium deficiency is a condition, mainly attributed to low selenium content in the soil or to long-term parenteral nutrition. Selenium is essential for several biochemical mechanisms and selenium blood decline concentrations relate to chronic age-related disease such as cancer, cardiovascular disease and immune dysfunctions (Seiler 2001). During ageing, selenium deficiency may occur in relation to intestinal malabsorption. However, few data report a marked selenium deficiency in old subjects (Seiler 2001). More recently, a paper has explored the relationships between plasma selenium and mortality in an elderly population for a long period of observation (9 years): the EVA (Etude du Vieillissement Artériel) study (Akbaraly et al. 2005). The authors have observed during this long period that the mortality rates were significantly higher in individuals with low selenium [1.01 µmol/L: a value below the cutoff considered as optimal (1.25–1.50 µmol/L; Thomson 2004; Combs 2001)]. When the underlying causes of death were considered, an association with low selenium and cancer-related mortality was found. The same authors suggest that plasma selenium could be an indicator of longevity in a preaging, independently living population not specifically at risk for cancer and cardiovascular diseases. Survival curves illustrate that the relationship between plasma selenium and mortality remained pertinent during the entire 9-year period (Akbaraly et al. 2005). However, the mechanism of this potential relationship is still under debate and further research needed especially on the role played by selenoproteins on this phenomenon. Other authors demonstrate selenium deficiency in elderly people in relation to hypothyroidism (Oliveri et al. 1996). Interestingly, human healthy centenarians display selenium values quite similar to normal elderly (Savarino et al. 2001). As few trials have been carried out up to date in elderly, it is difficult to report a specific beneficial effect of selenium in immunosenescence, even if beneficial effects of selenium supplementation on lymphocyte mitogen responsiveness have been reported in institutionalized elderly individuals (Peretz et al. 1991) and in old animals (Roy et al. 1995). Moreover, a Finnish study adding selenium to fertilizer has shown only an increased selenium status in the general population (young, adult, old; Aro et al. 1995), but not on its possible beneficial effects. The major evidence of the beneficial effects of selenium relate to age-associated diseases. Many studies have investigated the effects of selenium in carcinogen-exposed animals showing a reduction in tumor incidence and/or preneoplastic endpoints (Reid et al. 2002). A supplementation with 200 µg/day of organic selenium in randomized subjects showed preventive effects in the incidence and the mortality from various types of cancer (prostate, colorectal and lung cancer; Clark et al. 1998; Reid et al. 2002). Another large supplementation

trial in which a physiological amount of selenium (50 μg) was recently performed in Lixian (North China) in order to test its possible beneficial effect in preventing cancer. A small but significant reduction in total and cancer mortality was observed in subjects receiving selenium supplement. The reduction were shown to be greater in women than men and interestingly more pronounced in persons under the age of 55 years compared to individuals older than 55 years (Blot et al. 1995). Considering these results, it can be assumed that younger persons might be more amenable to a protective effect of selenium supplementation with thus a role of selenium in preventing age-related diseases or in enhancing the innate immune defenses in the course of the pathology, as observed in selenium supplemented cancer patients (200 mg/d of sodium selenite; Kiremidjian-Schumacher et al. 2000).

The relevance of selenium in the etiology of cardiovascular diseases has been also studied. Selenium metabolism is potentially involved in several protective biochemical pathways related to cardiovascular disease, such as reduction of LDL levels and lipoprotein oxidation, inhibition of foam-cell formation and shift in prostaglandin production from prostacyclin to tromboxane (Alissa et al. 2003). However, Wei et al. (2004) found no association between death for cardiovascular diseases and baseline selenium status in a cohort with a mean serum concentration of 0.93 μmol/L in younger individuals (mean age, 57 years). The major studies on the incidence of cardiovascular diseases in these last 5 years have been performed in adult people using a combinations of multivitamins and some trace elements, including selenium, as possible prevention of cardiovascular diseases (atherosclerosis, myocardial infarction, thrombosis). All these studies have shown a less incidence of cardiovascular diseases after supplementation with these combinations in comparison to placebo groups (Czernichow et al. 2005; Shenoy et al. 2006). Therefore, the existence of a clear link between selenium deficiency "in se" and cardiovascular disease remains to be clearly defined.

Finally, an intriguing point is the association between selenium deficiency, immune response and increased incidence of infections in adults and elderly. Patients with systemic inflammatory response syndrome display a strong impairment in immune efficiency, a decrease of 40% in plasma selenium concentrations coupled with increased morbidity and mortality rates (Forceville et al. 1998). The interrelationships between selenium deficiency, impaired immune response and infections have been clearly shown in experimental animals. An inoculated avirulent virus in selenium deficient animals turns into a virulent one due to genomic changes within the virus, provoking an impaired humoral immune defence (Beck 1999). In humans, a relevant clinical trial with multivitamins and selenium has shown an increment of CD4+ counts over the baseline levels (Coodley 1995) and enhanced GPx and GSH activity (Delmas-Beauvieux et al. 2006) in HIV infected patients This finding suggests that cysteine/GSH are effective natural inhibitors/combaters of (AIDS) viruses and thereby capable in preventing the development of chronic virus diseases that can lead to AIDS (Rayman 2000). Moreover, supplementation with multivitamins and trace elements, including Se, during treatment of pulmonary Tuberculosis may reduce mortality in subjects co-infected with HIV (Range et al. 2006). Therefore, an enhanced oxidative stress, caused by selenium deficiency, is the reason of

possible viral genetic changes (Beck et al. 2003) and increased progression of viral infections with subsequent impaired immune defense (Daniels 2004).

Additionally, it is also of interest for the role played by selenium deficiency in viral infections the following points: (i) the emergence during these last 4 years (from 2003) of a newly recognized human disease agent (coronavirus) that causes SARS from Guangdong Province of China (Lashley 2006) as well as from Northern Vietnam (Reynolds et al. 2006), where significant areas of overt selenium deficiency exist (Xia et al. 2005); ii) the increased risk of enhanced virulence of influenza virus in elderly (Ellis et al. 2003) associated with a possible selenium deficiency (Seiler 2001). Therefore, the selenium deficiency may be considered as a relevant risk factor for the appearance of age-related diseases (cancer, cardiovascular diseases and infections by viruses, which may become more virulent or mutated). Such a risk is of relevance in elderly because accumulating data suggest that persistent infection with Varicella-zoster virus (VZV; Arvin 1996), Epstein-Barr virus (EBV; Stowe et al. 2007) and particularly CMV (McVoy and Adler 1989) impacts upon the immune system in aging and may contribute to the immune risk phenotype (IRP), which predicts remaining longevity in the very elderly (Pawelec et al. 2005). Specific study on these aspects should be encouraged taking into account the possible relevant implications for public health.

3.4 Interrelationship Between Zinc and Selenium: Implications for Healthy Ageing

Dietary zinc and selenium are important nutritional factors for the immune response in protecting against the appearance of age-related diseases. The regulation of zinc ion bioavailability by selenium and selenoproteins has been recently investigated (Maret 2003). Zinc/thiolate coordination occurs in MT affecting the binding and release of zinc from MT. Zinc/thiolate cluster of MT can be oxidized by glutathion disulfide (GSSG) or other disulphides in order to release zinc. However, the efficiency of this chemical reaction seems very low even at high concentrations of GSSG in the absence of selenium. In contrast, the release of zinc from MT occur very rapidly following the addition of selenium compounds that has the capacity to form a catalytic selenol(ate), releases zinc (Maret 2003). The mechanism of the reaction was suggested to proceed through an activated selenenyl sulphide R-Se-S-G intermediate which, in turn, oxidizes the zinc-thiolate cluster of MT to form R-Se-S-MT with the concomitant release of zinc during the oxidation (Chen and Maret 2001). The selenol group is subsequently released by the attack of a nearby thiol group of MT that convert R-Se-S-MT into thionein generating a catalytic cycle of oxidative zinc release from MT. Other oxidized selenium compound, such as selenoxide and selenic acid may be directly reduced by MT through the formation of a R-Se-S-MT intermediates and the concomitant release of zinc, followed by the formation of an inter- or intramolecular disulfide bond (Chen and Maret 2001; Jacob et al. 1999; Klotz et al. 2003).

Selenium compounds also catalyze the release of zinc from MT in peroxidation and thiol/disulfide-interchange reactions. In presence of t-butylhydroperoxide, GPx catalyses the MT oxidation with subsequent zinc release, suggesting that MT may serve as reducing agents for GPx (or at least some GPx isoforms) in alternative to GSH (Jacob et al. 1999).Therefore, the assessment of zinc ion bioavailability, MT and selenium concentrations could represent useful tools for studying the physiology of successful ageing. Indeed, a recent study shows that 84.4% of the 'healthy' nonagenarian/centenarians display both zinc and selenium levels equal or greater than the lowest values in the elderly (Savarino et al. 2001). Moreover, healthy nonagenarians display low MT, good zinc ion bioavailability (Mocchegiani et al. 2002a) and satisfactory GPx activity (Mecocci et al. 2000). These findings suggest that an adequate zinc and selenium content in cells and tissues are crucial to achieve health ageing and longevity. In this context, Girodon et al. (1999) determined the effects of a long-term (for 2 years) daily supplementation with zinc (20 mg) plus selenium (100 μg) on immunity and the incidence of infections in a large number (n.725) institutionalized elderly people (> 65 years). The main results of the study were: (1) selenium deficient patients decreased from about 80% to 5–10% in the selenium supplemented group after 6 months of supplementation with respect to placebo group; (2) antibody titres after influenza vaccine were higher in groups that receive trace elements; (3) trace element supplemented patients were those who remained most free of respiratory tract infections than placebo group. These findings suggest that low dose supplementation of zinc and selenium provides significant improvement in elderly patients by increasing the humoral response after vaccination and decreased influenza compliances (respiratory tract infections) with thus possible achievement of health longevity.

4   Conclusions and Future Remarks

Beneficial effects obtained by zinc and selenium supplementation alone or associated on immune response and at clinical level are summarized in Table 1. Therefore, even if some controversial finding exists on the "real" necessity of micronutrient supplementation (Dangouret al.2004), the huge amount of data reported associated to observational data, clearly suggests that zinc and selenium play a pivotal role for immunosenescence in order to achieve healthy ageing and longevity. However, zinc seems to plays the major role because some biochemical mechanisms involved in the action of selenium are under the control of zinc ion bioavailability, which in turn is affected by MT and ZnT expression. One of the most relevant biochemical pathways is the release of zinc by MT through interactions with GPx and intracellular disulphides. However, some points require further investigations. First of all, the reason of a possible limited zinc release in ageing and the biochemical mechanism involved, in particular addressing NO-related intracellular pathways. Such an investigation is relevant taking into account the double face of NO action: or as antioxidant or as inducer of cell death (Colasanti and Suzuki 2000). Although useful tools are now available, such

Table 1 Possible causes of zinc and selenium deficiency in ageing and the main positive immune and clinical/biochemical effects of the related supplementation in experimental animals, in elderly and in syndrome of premature ageing (Down's syndrome)

Micronutrients	Possible causes of micronutrient deficiency in ageing	Immune and clinical/biochemical positive effects of micronutrient/s supplementation
Zinc	Frequent deficiency due to low dietary intake, enhanced urinary excretion, intestinal malabsorption. A limited zinc release from MT has been also proposed.	1. Enhanced NK cell cytotoxicity, cell-mediated immune response and thymulin activity; increased IFN-γ production, reduced levels of activated T helper cells; improved response to skin-test antigens and taste acuity 2. Lowering of plasma lipid peroxide levels 3. Restoration of TH1/TH2 paradigm 4. Increased ZnT1 and ZnT expression in lymphocytes 5. Reduced incidence of infection relapses in elderly, old infected patients and Down's syndrome subjects 6. Marginal effects on copper levels 7. Increased rate of survival in old, nude and thymectomized mice 8. Preservation of liver NKT γδ cells in old mice 9. Preservation of liver NKT cell cytotoxicity in old mice 10. Inducing apoptosis of only damaged cells in presence of high oxidative stress
Selenium	Decline with age mainly due to intestinal malabsorption	1. Increased lymphocyte mitogen response 2. Increased IL-2 receptor expression 3. Increased neutrophil function 4. Increased NK cell cytotoxicity 5. Decreased lung, colorectal and prostate cancer incidence 6. Lowering of cancer mortality 7. Less incidence of cardiovascular diseases 8. Decreased virulence of ECV, CBV and CMV
Selenium plus zinc		1. Improvements of antibody titres after influenza vaccination 2. Decreased influenza compliances (respiratory tract infections)

as NO donors and zinc fluorescent probes (zinpyr-1 and fluozin-3), in order to test the capacity of the cells in the zinc release by MT, the quantity of labile intracellular zinc in old age remains to be furtherly explored. This last point is also crucial because a fine modulation of intracellular labile zinc is fundamental in order to avoid an excessive zinc release by MT that can result toxic for the cell with subsequent cell-death. Moreover, the association of these studies with the role played by ZnT in ageing may give a more exhaustive picture of the role played by zinc in ageing. The results may

form a rationale to select old individuals who effectively need zinc supplementation because zinc, in a some extent, may be also toxic for the immune system leading to a further worsening of the already dysfunctional immune functions in ageing. In fact, many clinical trials of zinc supplementation in elderly report contradictory data on the benefit of zinc supplementation upon the immune functions. Thus, it is necessary to have many useful tools to screen real zinc-deficient old subjects. Among these tools, the genetic screening for some polymorphisms of MT, such as MT1A, might constitute a useful additional value in screening old subjects healthy ageing and longevity. Indeed, old subjects noncarriers of the C allele for MT1A +647 polymorphism display a better preservation of intracellular zinc homeostasis at advanced age, less inflammation, and are predisposed to the longevity with respect to old subjects carrying the C allele for the same MT polymorphism. This finding further suggests that only a certain number of old subjects are prone to zinc supplementation, and not all old population. In the case herein reported, a simple genotype screening might be useful to check the old subjects who should more frequently assess their zinc status for a possible zinc supplementation. In this context, genetic studies and the effect of zinc supplementation exclusively in old subjects with determinate polymorphisms for MT and IL-6 are studied in ZINCAGE project (www.zincage.org) funded by European Commission (EC) in FP6. Another project funded by EC in FP5 (ZENITH) confirms the presence of defects in zinc status and immune response in elderly. However, the biology of zinc is very complex and further studies are necessary in ageing especially addressed to the zinc-binding proteins strictly related to the inflammation and oxidative stress because both these conditions are the basis for the onset of a possible zinc dyshomeostasis in elderly (Mocchegiani et al. 2006).

With regard to selenium, the mechanisms of action of Se through selenoproteins against oxidative damage have been clear established, even if some aspects at genetic level especially regarding to the glutatione peroxidases require further studies. Indeed, while on one hand the genomic sequence of all GPxs isoforms has been established, the evolutionary reasons of an incorrect splicing of the selenium-independent GPx5 in humans is still to investigate. Anyway, selenium through selenoproteins has a wide range of action affecting the antioxidant system, the thyroid hormones turnover and the immune functions with a special focus on innate immune response. On the other hand, a correct thyroid hormone turnover affects the immune performances (Mocchegiani et al. 2006). As such, selenium treatment has been performed in various pathologies characterized by selenium deficiency, high oxidative stress and impaired immune function, such as cancer, infections, cardiovascular diseases as well as ageing. In this context, the more intriguing finding is the discovery that selenium deficiency in the diet or in soil is implicated in the mutation of a normally avirulent B3 coxsackievirus (CBV3/0) into a virulent virus (CBV3/20) by inducing changes in viral genoma. Moreover, a marginal selenium deficiency (1.10 μmol/L) causes a higher rate of mortality (by cancer) in old people with respect to old individuals with baseline selenium values (1.25 μmol/L). Therefore from the review data herein reported, zinc and selenium in the daily diet during ageing may be relevant in order to preserve immune and antioxidant functions, which can lead to healthy ageing and longevity. Alternatively, a

combined oral supplementation of these micronutrients can be recommended taking into account the beneficial effects of zinc and selenium in improving the humoral immune response in old vaccinated individuals (Duchateau et al. 2004; Girodon et al. 1999). However, the gap between the estimated average requirement of zinc and the upper limit of safe intake is relatively narrow, because excessive zinc may be toxic (Fosmire 1990). Concerning to selenium, even if few reported cases have been associated with an excessive intake of selenium, it has to be taken into account that the Institute of Medicine of the National Academy of Sciences has set a tolerable upper intake level for selenium at 400 micrograms per day for adults to prevent the risk of developing selenosis (Johnson et al. 2003). Therefore, supplementation with zinc and selenium can be recommended in old people who effectively need zinc and/or selenium supplementation after a careful evaluation of the "zinc/selenium status" through plasma measurement, clinical features and possibly evaluating the intracellular content of zinc and selenium. The usefulness of MT polymorphisms in identifying subjects at risk for zinc deficiency might be an additional tool. As such, the impaired immune functions in elderly, through these two trace elements, may be restored with subsequent healthy ageing and longevity.

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