Review

Micronutrient (Zn, Cu, Fe)–gene interactions in ageing and inflammatory age-related diseases: Implications for treatments

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ABSTRACT

In ageing, alterations in inflammatory/immune response and antioxidant capacity lead to increased susceptibility to diseases and loss of mobility and agility. Various essential micronutrients in the diet are involved in age-altered biological functions. Micronutrients (zinc, copper, iron) play a pivotal role either in maintaining and reinforcing the immune and antioxidant performances or in affecting the complex network of genes (*nutrigenomic approach*) involved in encoding proteins for a correct inflammatory/immune response. By the other side, the genetic inter-individual variability may affect the absorption and uptake of the micronutrients (*nutrigenetic approach*) with subsequent altered effects on inflammatory/immune response and antioxidant activity. Therefore, the individual micronutrient–gene interactions are fundamental to achieve healthy ageing. In this review, we report and discuss the role of micronutrients (Zn, Cu, Fe)–gene interactions in relation to the inflammatory status and the possibility of a supplement in the event of a micronutrient deficiency or chelation in presence of micronutrient overload in relation to specific polymorphisms of inflammatory proteins or proteins related of the delivery of the micronutrients to various organs and tissues. In this last context, we report the protein–metal speciation analysis in order to have, coupled with micronutrient–gene interactions, a more complete picture of the individual need in micronutrient supplementation or chelation to achieve healthy ageing and longevity.

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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 inflammatory/immune response and antioxidant capacity play key roles. The inability of an old organism in remodelling these changes may lead to the appearance of some degenerative age-related diseases. As a result, the “remodelling theory of ageing” has been proposed (Paolisso et al., 2000). Various essential micronutrients in the diet are involved in the capacity of the organism in remodelling the altered biological functions (Alvarez-León et al., 2006). The dietary intake of essential micronutrients is usually inadequate in elderly (Ames, 2006) owing to different causes. 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). This gap is worsened by loss of appetite, lack of teeth, reduced mineral

intestinal absorption and decreased requirement of energy. The deficiency in micronutrients largely contributes to the age-related weakness of the immune functions and antioxidant defence by external noxae (Failla, 2003). On the other hand, many micronutrients provide, directly or indirectly, to the biological activity of some antioxidant enzymes to sustain the immune efficiency and to keep under control the inflammation. Consequently, many body homeostatic mechanisms can be preserved with subsequent achievement of the longevity (Mocchegiani et al., 2006). Longitudinal studies in human centenarians (successful ageing) show that a satisfactory content of some trace elements within the cells leads to good performances in several immune functions and in preserving a low grade of inflammation (Chernoff, 2001). In this context, micronutrients (zinc, copper, iron) play a pivotal role either in maintaining and reinforcing the immune and antioxidant performances or in affecting the complex network of genes (*nutrigenomic approach*) involved in encoding proteins with anti and pro-inflammatory tasks as well as in transporter proteins required for the metal distribution to various organs and tissues. Pro- and anti-inflammatory cytokines and the regulators of trace elements homeostasis, such as Metallothioneins (MT) and metal transporters, are of relevance taking into account that the same genes codifying for cytokines, MT and metal transporters are also involved, when mutated or related to their specific polymorphisms, to the susceptibility of the major

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geriatric pathologies, such as diabetes, dementia, cardiovascular diseases and infections (Mocchegiani et al., 2008c). At the same time, the genetic inter-individual variability may affect the absorption and uptake of the micronutrients (*nutrigenetic approach*) with subsequent altered effects on inflammatory/immune response and antioxidant activity. Therefore, current problems are to understand how the interactions between genetic factors and micronutrients (*nutrigenomic and nutrigenetic approaches*) may influence the ageing process in view of the high impact on gene expression, protein production and epigenetic mechanisms involved in the regulation of the longevity (Slagboom et al., 2011). In this context, some micronutrients can be also toxic depending on their intake of improper diet or on the genetic inter-individual variability (Mocchegiani et al., 2008c). Thus, this review reports the micronutrient (zinc, copper, iron)–gene interactions in ageing and their impact on the healthy status with a focus on the more precise determination of their specific transporter proteins, by means of the method of protein–metal speciation analysis (Sanz-Medel et al., 2003; Malavolta et al., 2012). As a result, the association between micronutrient–gene interactions and protein–metal speciation analysis may give a more complete picture of the individual need in micronutrient supplementation or chelation in order to reach healthy ageing and longevity.

2. Zinc and zinc-gene interaction

Zinc is a relevant trace element for many biological functions and involved in numerous cellular processes on the view of its occurrence in over three hundred enzymes as a catalytic metal and in many proteins as a structural metal. These pleiotropic actions require tight regulation in allocating zinc at the right time to the correct protein(s). Metallo-regulatory proteins perform this function. Thus, both the availability of zinc ions from the diet and the proper functioning of the proteins that handle zinc are critical for maintaining good health (Maret and Sandstead, 2006). Such a role is reinforced by the effect of zinc in genetic stability and gene expression, taking into account that about 25% of the cellular zinc content is incorporated into the nucleus (Cousins, 1998). Mutations in some genes coding for zinc-related proteins are the basis for inborn errors of zinc metabolism, as it occurs in acrodermatitis enteropathica: a rare human disorder with a mutation in the zinc transporter Zip4 (SLC39A4) resulting in severe zinc deficiency (Küry et al., 2002). Furthermore, the risk of type 2 diabetes has been associated with a single nucleotide polymorphism (SNP) resulting in an Trp325Arg substitution in the pancreatic Beta-cell-specific zinc transporter ZnT-8 (SLC30A8), which provides zinc for insulin maturation and/or storage (Sladek et al., 2007). Moreover, a novel Zip2 Gln/Arg/Leu codon 2 polymorphism is associated with carotid artery disease (CAD) (Giacconi et al., 2008b). Although the recommended daily zinc intake is between 10 and 40 mg/day (RDA), the dietary zinc intake and absorption are altered in ageing due to different causes: from psychosocial to subcellular factors, including alterations in zinc transporter proteins (Maret and Sandstead, 2006). In this context, the cellular zinc uptake is strongly reduced in ageing by altered gene expression of zinc transporters Zip1, Zip2, Zip3 (Giacconi et al., 2011) due to chronic inflammation or DNA methylation. This finding leads to consider zinc transporters as pivotal in affecting the genetic stability with subsequent modifications of the zinc homeostasis and metabolism. The discovery of cellular genomic instability in chronic inflammation owing to altered gene expressions of some zinc transporters (Zip14, Zip5, ZnT1, ZnT4, ZnT5, ZnT6) (Cousins, 2010), is in line with this interpretation. On the other hand, the influence of zinc on genomic stability is also supported by the involvement of zinc, via zinc finger motifs, in DNA replication of many proteins essential for cell

proliferation, cell differentiation, cell growth arrest, cell division, signal transmission, growth factor productions, protooncogene activations, chemokine productions as well as in codifying hormone nuclear receptor superfamily and in nuclear transcriptional factor activations (Table 1). In zinc-finger motifs, the metal provides a scaffold that organizes protein sub-domains for the interaction with either DNA or other proteins (Klug, 1999). The zinc-finger motifs, other than to modulate the gene expression of metallo-regulatory proteins, act also as “zinc sensors” for many transcriptional factors with also an autoregulatory mechanism (Lyons et al., 2000), as it occurs for various transcriptional factors (Egr-1, Sp1, A20, NF- κ B, STAT, HSF-1, Kruppel zinc-fingers family) during inflammation and after antigenic stimuli (Mocchegiani et al., 1998; Rink and Kirchner, 2000). Exhaustive reviews are available in literature regarding to the effect of these transcriptional factors in affecting zinc-gene interactions related to the inflammatory/immune response in ageing (Prasad, 2000; Vasto et al., 2006; Haase and Rink, 2009; Mocchegiani and Malavolta, 2008). Besides having a fundamental role in innate and cell-mediated immunity because involved in T-cell maturation, differentiation and proliferation at thymic and liver extrathymic levels (Mocchegiani et al., 2012), zinc plays a key role in adaptive immunity and, consequently, in inflammatory/immune response (Haase and Rink, 2009). Both in ageing and in zinc deficiency, the plasma concentrations of IL-6, IL-8, MCP-1, MIP-1 α and TNF- α display a progressive elevation. In particular, Th1 (IFN- γ , IL-2) cytokines decrease whereas Th2 (IL-4, IL-10) cytokines increase (Mariani et al., 2006) leading to an imbalance of Th1/Th2 paradigm with a shift towards Th2 production with subsequent chronic low grade of inflammation (named “inflammaging”) (Franceschi, 2007) and altered inflammatory/immune response (Uciechowski et al., 2008). In this context, the zinc-gene interaction is pivotal through the zinc finger motifs. Among them cited above, the Kruppel zinc-fingers (Klf) family has emerged as critical regulators of important functions all over the body because implicated, other than in inflammatory/immune response, also in cell proliferation, apoptosis and differentiation (Pearson et al., 2008). Their features are to mediate activation and/or repression of transcription. Within the family, Klf2 plays a peculiar role because involved, via NF- κ B, in IFN- γ and TNF- α production (Das et al., 2006), and therefore in keeping under control the inflammation. Klf2 plays also a role in T-cell differentiation, maturation and function. In absence of Klf2, mature and single positive T cells are more prone to undergo to apoptosis. Klf2 also regulates the migration of mature thymocytes from the thymus to the periphery (Pearson et al., 2008). These findings support the relevance of zinc in ageing both in preserving T cells by apoptosis and, together with the zinc-dependent thymic hormone thymulin (Mocchegiani et al., 1998), in the homing of mature T cells from the thymus into the circulation. Although other Kruppel-like zinc-finger motifs are involved in many body homeostatic mechanisms, Klf5 is crucial because affecting the nuclear enzyme PARP-1 (Swamynathan, 2010), which is in turn involved in DNA-repair during oxidative stress and inflammation in ageing and age-related diseases (Mocchegiani et al., 2000). Therefore, zinc-finger proteins are indispensable to transduce the signal from the receptors to gene expressions, via transcriptional factors, resulting the pillars for a good functioning in the physiological cascade of the zinc-gene interactions for the whole life of an organism.

Other than DNA zinc-finger motifs, zinc affects a wide range of genes (assayed by DNA array and elaborated with Ingenuity Pathway Analysis™ or by Northern blot) responsible of many biological functions, including metabolism, oxidative stress, inflammatory/immune response, biotransformation, protein degradation, DNA-repair and food intake (Table 2). Several canonical pathways were significantly regulated by zinc in young and elderly donors as

Table 1

Structural and catalytic action of zinc within ‘zinc fingers’ and proteins, compounds or peptides involved in DNA replication and other relevant biological functions.

Gene symbol/abbreviation	Full name	Biological function
SP1	Specificity protein1	<i>Cell proliferation</i> (Prasad, 2000; Verstrepen et al., 2010; Philipsen and Suske, 1999; Lhomond et al., 1996; Zhang et al., 2011; Haase and Rink, 2009)
ALR	Augmenter of liver regeneration	
MMPs	Matrix metalloproteases	
BP10	Blastula protease 10	
BP1	Beta protein 1	
STAF50	Stimulated trans-acting factor 50	
TIMPs	Tissue inhibitors of metalloproteases	
DFP2	Dark foot pads 2	
A2M	α -2 macroglobulin	
A-20	Tumor necrosis factor alpha-induced protein 3	
GATA family	GATA family of transcription factors	
MP1	Mitogen Protein1	
ITGA2B	platelet glycoprotein IIb of IIb/IIIa complex	
IKAROS family	IKAROS family of zinc fingers	
ZEB1	Zinc finger E-box binding homeobox 1	
TGF- β	Transforming Growth Factor-beta	<i>Cell differentiation</i> (Payne, 2011; Bates et al., 2008; Vinayagam et al., 2011)
CCNT1	Cyclin T1	
PKC	Protein kinase-C	
ZNF162	Zinc finger protein 162	
EGF	Epidermal Growth Factor	
IL-1	Interleukin-1	
IL-2	Interleukin-2	
IL-4	Interleukin-4	
IL-5	Interleukin-5	
IL-6	Interleukin-6	
IL-7	Interleukin-7	
IL-12	Interleukin-12	
IFN- α	Interferon-alpha	
TNF- α	Tumor Necrosis Factor alpha	
TP53	p53 tumor suppressor	<i>Cell growth arrest</i> (Vinayagam et al., 2011; Prasad, 2000) <i>Cell division</i> (Vinayagam et al., 2011; Mocchegiani et al., 1998) <i>Signal transmission</i> (Haase and Rink, 2009; Mocchegiani et al., 1998)
CDKN1A	cyclin-dependent kinase inhibitor 1A	
MYC	Avian myelocytomatosis viral oncogene homolog	
FOS	FBJ murine osteosarcoma viral oncogene homolog	
JUN	Jun proto-oncogene	
BCL2	B-cell CLL/lymphoma 2	
EVI 1	ecotropic virus integration site 1	
GFI-1	Growth Factor Independent 1	
HIC-1	Hypermethylated In Cancer 1	
RANTES	Chemokine (C-C motif) ligand 5	
MCP-1	Monocyte Chemotactic Protein-1	
NF-kB	Nuclear Factor-kB	
AP-1	Activator Protein-1	
EGR-1	Early Growth Response1	
EGR-2	Early Growth Response 2	
WT-1	Wilms Tumor 1	<i>Protoncogenes for prevention or activation apoptosis</i> (Vilborg et al., 2010; Mocchegiani et al., 1998; Hainaut and Mann, 2001; Wieser, 2007)
TFIIIA	Transcription Factor IIIA	
TRAF-2	TNF Receptor-Associated factor 2	
ZNF382	Zinc finger protein 382	
RREB1	Ras responsive element binding protein 1	
KLF family	Kruppel zinc fingers family (Klf) 1-2-3-4-5-6-7-9-10-11-13-15-16)	
GHR1A	Growth hormone receptor 1A	
GR	Glucocorticoid receptor GR	
PGE2R	Prostaglandin E2 receptor	
IGF-1R	Insulin like Growth Factor-1 receptor	
PPAR γ	Peroxisome Proliferator-Activated Receptor γ	

well as in zinc deficiency (Mazzatti et al., 2007; tom Dieck et al., 2003). Among them, PPAR α gene is very sensible to zinc signalling. It is highly expressed in young age, low expressed in elderly, strictly related to zinc deficiency and associated with impaired SOD gene expression leading to increased ROS production with subsequent appearance of degenerative diseases, such as cancer (Meyer et al., 2002). “*In vitro*” zinc induces an upregulation of PPAR α gene via inhibition of NF-kB (Mazzatti et al., 2007), suggesting that the modulation of PPAR α gene expression by zinc in elderly may counteract increased inflammation and oxidative stress. Moreover, zinc (32 μ M) also blocks G2/M cell cycle in precancerous human bronchial epithelial cells upregulating p53 and p21 gene expressions (Wong et al., 2008).

From this plethora of data, it emerges the peculiar role played by zinc in affecting the gene expressions related to the inflammation, oxidative stress and cell cycle. In order to regulate the activity of this complex intracellular zinc-gene network, sophisticated mechanisms exist. In this context, Metallothioneins (MT) are essential to intracellular zinc homeostasis by sequestration and release of the metal at the occurrence, via Nitric Oxide nitrosylation (Spahl et al., 2003), and thereby in controlling the available free zinc ions (Palmiter, 1998) and, consequently, in affecting the zinc-gene interactions (Maret et al., 1999). MT are polymorphic proteins, and MT-I and MT-II isoforms are the best known and studied (West et al., 1990). In chronic stress and inflammation, such as in ageing, high MT are less capable in zinc release with subsequent low zinc

Table 2

Encoded proteins affected by zinc-gene interactions determined by DNA Array and elaborated by Ingenuity Pathway Analysis™ (A) or by Northern blot (B).

Encoded proteins		Function
Peroxisome proliferative activated receptor, alpha-gamma (a)	A	Lipid metabolism (a), molecular transport and binding (b), Stress response (c), Biotransformation and detoxification (d)
Lipoprotein lipase (LPL) (a)	B	
Carboxylesterase (a)	B	
Lysophospholipase (a)	B	
3-Keto acyl-CoA thiolase 2 (a)	B	
Acetyl-CoA acyltransferase 2 (a)	B	
Glycerol kinase (a)	B	
Lipogenic factor (S14 gene) (a)	B	
Fatty acid-binding protein (a)	B	
Fatty acid transporter (a)	B	
Regucalcin (senescence marker protein-30) (RGN) (b)	A	Cell-to-cell signalling and interaction (e), Cellular movement (f), Intracellular trafficking (g), Signal transduction (h)
Laminin, beta 3 (LAMB3) (b)	A	
Phosphatidylserine binding protein (SDPR) (b)	A	
Cryptochrome 2 (photolyase-like) (CRY2) (b)	A	
Protein Kinase C (b)	B	
Hemoglobin, alpha 1 (HBA1) (b)	A	
K voltage-gated channel, shaker-related subfamily, (KCNA1) (b)	A	
Glutathione peroxidase (c)	B	
Glutathione transferase subunit 5 (c)	B	
Superoxide dismutase-1 (SOD-1) (c)	B	
Metallothionein 1 (MT1)	A	
Cytochrome P450 (CYP4B1) (d)	B	
Cytochrome P450 (CYP4A3) (d)	B	
Cytochrome P450 (CYP2C23) (d)	B	
NADPH-cytochrome P450 reductase (d)	B	
Liver aldehyde oxidase (d)	B	
Thyrosine sulfotransferase (d)	B	
UDP-glucuronosyltransferase (d)	B	
Alkaline phosphatase, intestinal (ALPI) (d)	A	
Matrix metalloproteinase 19 (ADAM19) (e)	A	
Prostaglandin-endoperoxide synthase 1 (e)	A	
Matrix metalloproteinases (MMP 2,3,7,9,19) (e)	A	
Tissue factor pathway inhibitor 2 (TFPI2) (e)	A	
Angiopoietin 1 (ANGPT1) (e)	A	
Proteinase 3 (PRN3) (e)	A	
Pyrimidinergic receptor P2Y, G-protein coupled, 6 (P2RY6) (e,h)	A	
Integrin signaling (ITG) (e)	A	
Ras protein-specific guanine nucleotide-releasing factor 2(RASGRF2)(e)	A	
Spondin 1, extracellular matrix protein (SPON1) (e)	A	
Integrin, beta 8 (ITGB8) (e)	A	
Chemokine (C-X-C motif) ligand 3 (CXCL3) (f)	A	
Connective tissue growth factor (CTGF) (f)	A	
Ribosomal protein S15 (RPS15) (f)	A	
Death inducer-obliterator 1 (DIDO1) (f)	A	
Syntaxin (2,4) (g)	B	
Synapsin II (g)	B	
Fatty acid binding protein 4, adipocyte (FABP4) (g)	A	
Torsin family 1, member A (torsin A) (TOR1A) (g)	A	
Arrestin D (h)	B	
Nuclear receptor subfamily 1, group H, member 3 (NR1H3) (h)	A	
B2 bradykinin receptor (h)	B	
IGF-1 (i)	B	Cellular growth and proliferation (i) Immune response (l) DNA-repair (m), Protein degradation (n)
IGF-binding protein 1 (i)	B	
IGF-binding protein 2 (i)	B	
IGF-binding protein, acid-labile subunit (i)	B	
Thyroxine deiodinase type I (i)	B	
Neurexophilin 4 (NXPH4) (i)	A	
Nerve growth factor (NGF) (i)	B	
Solute carrier family 14 (urea transporter), member 2 (SLC14A2) (i)	A	
Solute carrier 30 (zinc transporters ZnT Family) (i)	B	
Solute carrier 39 (zinc transporters Zip Family) (i)	B	
Leptin (obesity homolog, mouse) (LEP) (i)	A	
Phosphogluconate dehydrogenase (PGD) (i)	A	
Interleukin 1 receptor, type II (IL1R2) (l)	A	
Interferon, gamma-inducible protein 30 (IFI30) (l)	A	
Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61) (ITGB3) (l)	A	
Interleukin 24 (IL24) (l)	A	
Interleukin 6 (IL-6) (l)	A	
KIT ligand (KITLG) (l)	A	
Interferon beta 2 (l)	A	
Interleukin-2 (IL-2) (l)	A	
Tumour Necrosis Factor- α (TNF- α) (l)	A	
Interferon ($\alpha\beta\gamma$) receptor 1 (IFNAR1) (l)	A	
Indoleamine-pyrrole 2, 3 dioxygenase (INDO) (l)	A	

Table 2 (Continued)

Encoded proteins	Function
AP endonuclease (m)	B
Poly [ADP-ribose] polymerase 1 (PARP-1) (m)	B
Serpin peptidase inhibitor family (2,8,9) (n)	A
Cathepsin L-like 3 (CTSL) (n)	A
Cathepsin H (n)	B
Acyl-peptide hydrolase (n)	B
Ubiquitin-like protein (n)	B
Serine dehydratase (n)	B
Chloride ion Channel p64h1 (Intracellular ion channel)	B
Nap57 (rRNA biogenesis)	B
p53 and p21 (G2/M cell cycle arrest)	B
Propyl 4-hydrolase (Collagen metabolism)	B
FOF1-ATP Synthesis (subunit c) (ATP synthesis)	B
5-Aminolevulinic synthase (Porphyrin synthesis)	B
Ceruloplasmin (Fe II oxidation)	B
Neuropeptide Y (Food intake)	B
Bile acid CoA ligase (Bile acid conjugation)	B
Glucose-6-phosphatase (carbohydrate metabolism)	B
Galactose-1-phosphate uridylyl transferase (carbohydrate metabolism)	B
Transcriptional repressor NAB1 (Transcription)	B

Capital letters (A, B) represent the elaboration by which the zinc-gene interactions were determined with DNA array (A, elaboration by Ingenuity Pathway Analysis™; B, elaboration by Northern Blot). Lower letters (in brackets) in the right panel of the table represent the specific biological function of the respective corresponding encoded proteins reported in the left panel of the table.

Key references: Mazzatti et al., 2007, 2008; tom Dieck et al., 2003; Fukada et al., 2011; Blanchard et al., 2001; Mocchegiani and Malavolta, 2008.

ion bioavailability to fight stress and to support immune response (Mocchegiani et al., 2008c). As a consequence, many genes that act as “zinc sensors” are altered in ageing (Fülöp et al., 2003). In mammalian cells, the metal responsive transcription factor 1 (MTF-1) is an important zinc sensory transcriptional factor. MTF-1 binds to zinc-sensing gene promoter elements (MRE), and induces the gene transcription of key target genes, including MT-I and MT-II isoforms. The disruption of MTF-1 gene in mice is lethal (Andrews, 2001), suggesting that MTF-1 gene is fundamental for MT gene expression and the regulation of many zinc-target genes, including those ones related to inflammation (IL-6, TNF- α , Rantes, IL-8, MCP-1, PPAR α , PPAR γ) (Mazzatti et al., 2007). This finding is of a great relevance mainly for the very old age because in centenarians the MT gene expression is relatively low and coupled with good intracellular zinc ion bioavailability and low grade of inflammation with respect to normal ageing (Moroni et al., 2005), supporting the beneficial role of MTF-1/MT/zinc/gene interaction to achieve successful ageing. A novel polymorphism of MT1A (A/C (Asparagin/Threonin) transition at +647 position), coupled with a better zinc ion bioavailability and longevity in old women, confirms this assumption (Cipriano et al., 2006).

Another attractive point is the interaction between zinc and albumin, which is a zinc-binding protein in moving zinc within the body to various organs and tissues (Cousins, 1986). A lack of albumin in the urine (albuminuria) during ageing, other than inducing zinc deficiency, provokes sarcopenia (Visser et al., 2005) nephropathy (Martin and Sheaff, 2007) and mortality (Sahyoun et al., 1996). A recent study reports that polymorphisms of some genes, such as MYO16 (myosin heavy chain Myr 8), IRS2 (insulin receptor substrate 2), NEGR-1 (neuronal growth regulator-1), are associated with increased albuminuria and nephropathy in ageing (Tsaih et al., 2010). Hence, directly or indirectly via albumin, the relevance of the zinc-gene interactions in ageing is pivotal, with thus the suggestion of zinc supplementation in order to compensate the lack of zinc and, concomitantly, to achieve the healthy status. However, caution is required because abnormal zinc assumption may be toxic triggering apoptosis with deleterious side effects in various systems, organs and tissues (Sandstead, 1995). Accumulation of zinc and zinc toxicosis may also occur due to air zinc exposure (zinc oxide) (Plum et al., 2010), but especially by an incorrect dietary zinc assumption because leading to an imbalance with copper (Maret,

2006). Consequently, an alteration in zinc-gene interactions and the possible appearance of autoimmune phenomena or neurodegeneration occur (Mocchegiani et al., 2006), with thus the necessity to use chelating agents in presence of zinc toxicosis.

3. Zinc supplementation by zinc deficiency

During the last two decades, a lot of papers reports the beneficial effects of zinc supplementation upon the immune system and against oxidative stress in old mice and in elderly with also a role in prolonging the rate of survival in old mice because of a strong reduction (50%) of deaths by infections (Mocchegiani et al., 2007). However, contradictory data exist on the beneficial effects of the zinc supplementation in old people (Haase and Rink, 2009). The discrepancies among various authors are largely due to various factors, among them the different doses of zinc used, the length of the zinc supplementation, and the dietary habits are pivotal. From these studies, a physiological dose of zinc (15–40 mg/day as suggested by RDA) applied for a long periods (6–12 months) or high doses of zinc (100–200 mg/day) for short periods might induce limited effects on the immune response due to the zinc accumulation in immune cells and in various organs and tissues with subsequent toxic effect of zinc upon the immune functions (Sandstead, 1995). Indeed, high doses of zinc can trigger apoptosis in presence of high oxidative stress and inflammation (Fraker and Lill-Elghanian, 2004). Therefore, caution is advised for the management of zinc supplementation with the suggestion to perform the trial for short periods and on alternate cycles, as carried out in Down's syndrome subjects with subsequent beneficial effect of zinc upon the immune functions (Franceschi et al., 1988). However, one possible cause of the existing discrepancies may be the choice of old subjects who effectively need zinc supplementation in close relationship with the dietary habits and inflammatory status (i.e. IL-6 values). This assumption is supported by the discovery that old subjects carrying GG genotypes (termed C– carriers) in IL-6-174C/C locus display increased IL-6 production, low intracellular zinc ion availability, impaired innate immune response and enhanced MT. By contrast, old subjects carrying GC and CC genotypes (termed C+ carriers) in the same IL-6-174 locus display satisfactory intracellular zinc as well as good innate immune response (Mocchegiani et al., 2008b). But, the more intriguing finding is that male

Table 3Effect of zinc supplementation^a in elderly selected on the basis of MT and IL-6 polymorphisms and zinc status^b (data obtained from Zincage project).

Condition/target	Parameter	Effect (ref.)
Zinc status	Plasma zinc	↑ (Mocchegiani et al., 2008b)
	Plasma Zinc/albumin	↑ (Mocchegiani et al., 2008b)
	Labile intracellular zinc	↑↑ (Mocchegiani et al., 2008b)
	Metallothioneins	↑ (Mocchegiani et al., 2008b)
	NO-induced release of zinc	↑↑ (Mocchegiani et al., 2008b)
	Granulocyte zinc	↑↑ (Mocchegiani et al., 2008b)
	MT glutathionylation	– (Casadei et al., 2008)
Stress-related proteins	Poly(ADP-ribosyl)ation capacity	↑ (Kunzmann et al., 2008)
	ROS production	↓ (Mocchegiani and Malavolta, 2008)
	ApoJ plasma	–↑ (Mocchegiani et al., 2007)
	Genes involved in nitrosative stress (ATF2, CSF2, FOS, ICAM1, JUN, LTA, CCL2, SELE, VCAM1, iNOS, TNF, NFKB1)	↓ (Mazzatti et al., 2008)
	Total intracellular carbonyl levels	↓ (Cabreiro et al., 2008)
	MsR activity and protein expression	↑↑ (Cabreiro et al., 2008)
	Chymotrypsin-like peptidase activity of proteasome and 20S protein expression	↑ (Cabreiro et al., 2008)
	Chaperone (Hsp72) protein levels	–↑ (Putics et al., 2008)
	Chaperone (Hsp72) inducibility	↑↑ (Putics et al., 2008)
	Plasma SOD	↑ (Mariani et al., 2008a)
Antioxidant plasma enzymes	Erythrocyte SOD	↑ (Mariani et al., 2008a)
	Catalase	↓ (Mariani et al., 2008a)
	Glutathione peroxidase	↓ (Mariani et al., 2008a)
Thymic output	T-cell receptor excision circles (TRECs)	↓↑ (Final report of Zincage) (Mocchegiani et al., 2008a)
Senescence and apoptosis	Telomere length	–↑ (Canela et al., 2007)
	Early spontaneous apoptosis	↓ (Ostan et al., 2006)
	Late apoptosis	↓ (Ostan et al., 2006)
	Oxidative stress-induced apoptosis	↓ (Ostan et al., 2006)
	Mitochondrial membrane depolarization during spontaneous and dRib induced apoptosis	↓ (Ostan et al., 2006)
	Cell cycle	– (Ostan et al., 2006)
	IL-6, IL-8, MIP-1α	–↑ (Mariani et al., 2008b)
Plasma cytokines and chemokines	MCP-1, RANTES	– (Mariani et al., 2008b)
	NK lytic activity	↑↑ (Mariani E et al., 2008b)
Immune functions	Basal IFN-γ, IL-8, IL-1ra and IL-6 production	↓ (Uciechowski et al., 2008)
	Basal IL-10 and TNFα production	↓ (Uciechowski et al., 2008)
	Stimulated IFN-γ, IL-6, TNF-α, IL-1ra and IL-10 production	↑ (Varin et al., 2008)
	IL-2 and IL-6 STAT3 and STAT5 activation	– (Varin et al., 2008)
Jak/Stat signalling and immunomodulation	Activation-induced cell death (AICD)	↑ (Varin et al., 2008)
	Cytokines and metabolic gene expression response to zinc	↑↓ (Mazzatti et al., 2007)
	Activated T cells (CD3+ CD25+)	↓ (Varin et al., 2008)
T cells subsets	CD4:CD8	– (Malavolta et al., 2008)
	Frequencies of CMV-specific cells	– (Malavolta et al., 2008)
Cognitive functions	Perceived stress scale	↓ (Marcellini et al., 2008)

↑↑, strongly increased; ↑, increased; –, not modified; –↑, slightly increased; ↓↑, intervariability; ↓, decreased.

^a The dose of zinc used was 10 mg/day of zinc aspartate (Unizink 50, Kohler Pharma Corp., Alsbach-Hahnlein, Germany) for 45 days.^b Zinc status ≤10 μM (All the references come from the Zincage Consortium).

C+ carriers are more prone than C– carriers to reach centenarian age. Therefore, old C– carriers are likely to benefit more from zinc supplementation than old C+ carriers. Zinc supplementation in old C– subjects restores NK cell cytotoxicity to values present in old C+ carriers and considerably improves the zinc status (assessed by increments of intracellular Zn) and stress response (assessed by increments of ApoJ protein) (Mocchegiani et al., 2007). When the genetic variations of IL-6 polymorphism are associated with also the variations of MT1A +647A/C gene, the plasma zinc deficiency and the altered innate immune response is still more evident (Mocchegiani et al., 2008b), 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 with thus a precise and personalized zinc dietary intake or zinc supplementation. Such a role played by the genetic background is also evident in keeping better under control pro-inflammatory cytokine and chemokine productions after zinc supplementation (Mariani et al., 2008a,b) as well as in reducing the gene expression of IL-1

and its receptor (Mazzatti et al., 2008). A comprehensive portrait of the effect of zinc supplementation on zinc state, immune response, cytokines, chemokines and stress-related proteins in old people selected according to IL-6 and MT polymorphisms is provided in Table 3.

4. Zinc chelation by zinc toxicity

Despite the beneficial effect of zinc may be related to the choice of the subjects on the basis of their specific genetic background related to IL-6 and MT1A polymorphisms, an excess of zinc may be toxic because mainly inducing copper deficiency. Multiple adverse effects include decreases in copper-dependent enzymes (SOD), ceruloplasmin, cytochrome C oxidase, changes in immunological parameters, cholesterol and its lipoprotein distribution (Maret, 2006). The mechanisms by which an excess of zinc leads to copper deficiency are multiple. One of them is the reduction of gene expression of ATP7A protein appointed to the absorption of copper on the basolateral surface of enterocytes, as it occurs for example in Menkes' disease (Llanos and Mercer, 2002). But, the more intriguing

Table 4

Effect of zinc chelators as potential therapeutic strategy in presence of zinc toxicosis.

Chelator	Target	Model	Treatment	Effect	Reference
TPEN	A β deposits	Brain AD at post-mortem (in vitro model)	2 mM	Resolution	Cherny et al. (1999)
TPEN	TNF α , IL-13, IL-4 production	airway inflammation (animal model)	One i.p. injection (10 mg/Kg)	↓	Fukuyama et al. (2011)
TPEN	Neuronal cell death	Neuronal cultures by NO exposure (SNOC1-50 μ M) (in vitro model)	20 μ M	↓	Bossy-Wetzel et al. (2004)
TPEN	TNF- α , IL-6, IL-1 β production	Human monocytes after LPS stimulation for 24 h (in vitro model)	10–50 μ M for 30 min	↓	Haase et al. (2008)
TPEN	Apoptosis of cancer cells	MCF7 cells (in vitro model)	10 μ M for 48 h	↑	Hashemi et al. (2007)
Clioquinol	A β accumulation	APP2576 mice (animal model)	Oral administration 30 mg/kg/day for 9 weeks	↓	Cherny et al. (2001)
Clioquinol	A β ₄₂ accumulation	Alzheimer (human)	375 mg/day for 25 weeks	↓	Ritchie et al. (2003)
PBT2 (second generation of Clioquinol)	Cognitive functions A β ₄₂ accumulation	Alzheimer Phase II study (human)	250 mg/day for 12 weeks	↑	Lannfelt et al. (2008)
PBT2 (second generation of Clioquinol)	Synaptic plasticity	Tg2576 mice (animal model)	30 mg/kg/day for 11 days	↓ ↑	Faux et al. (2010) Adlard et al. (2011)
EDTA	Learning memory	HeLa cells exposed to toxic zinc (≥ 50 μ M) (in vitro model)	2 mM	↑	Wang et al. (2006)
EDTA	N-methylpurine-DNA glycosylase (MPG)	Human old lymphocytes (in vitro model)	0.5 mM for 24 hr	Blocked inhibition of MPG by zinc favouring DNA-repair ↓	Giacconi et al. (2011)
1,10-Phenanthroline	A β ₄₂ accumulation	APP overexpressing cells (in vitro model)	25 μ M	↓	Caragounis et al. (2007)
1,10-Phenanthroline	D1 cyclin and KFL4	Cancer cell proliferation (in vitro model)	50 μ L	↓	Huesca et al. (2009)

↑, Increase; ↓, decrease.

mechanism is the involvement of MT. Indeed, excess of zinc induces the synthesis in enterocytes of MT, which bind copper with a higher affinity than zinc with subsequent excretion of copper-bound MT in the faeces reducing the amount of copper delivered to the enterocyte (Webb and Holt, 1982). As such, a strong imbalance of the Zinc/Copper ratio may arise with subsequent disability and mortality in elderly (Malavolta et al., 2010; Mocchegiani et al., 2011). Excess of zinc ($\geq 50 \mu\text{M}$) may be also implicated in carcinogenesis because inhibiting the activity of some proteins (N-methylpurine-DNA glycosylase (MPG) and DNA ligase-1) involved in DNA repair of damaged proteins favouring mutagenesis, which is in turn blocked by adding EDTA in the culture medium (Yang et al., 1996; Wang et al., 2006). Moreover, zinc chelators TPEN or DPTA in MCF7 cancer cells increase apoptosis of cancer cells (Hashemi et al., 2007). However, an excess of zinc has the major effect on the brain with the appearance of neurodegenerative disease or neuronal injury (cerebral ischemia, epilepsy, brain trauma). Toxic zinc accumulation may result from either trans-synaptic zinc movement or mobilization from intracellular sites (Zn flux through receptor associated calcium channels, voltage-sensitive calcium channels) or Zn-sensitive membrane transporters (Weiss et al., 2000). Recent findings have suggested that postsynaptic zinc-sequestering proteins may represent the main sources of toxic zinc ions (Frederickson et al., 2004). MT-III isoform in the brain, which sequesters and rapidly releases zinc after oxidative stimuli (Chen et al., 2002), is a likely source of injury-mobilized zinc by MT-III in neurons after an excitotoxic insult (Lee et al., 2003). Zinc exerts its neurotoxicity enhancing the ROS production by mitochondria and the activation of apoptotic processes (Sensi and Jeng, 2004). These phenomena occur in age-related neurodegenerative diseases, such as Alzheimer type dementia (AD), in which zinc may also favour the accumulation of β -amyloid ($\text{A}\beta$) in senile plaques (Bush, 2000). This last protective role of zinc because precluding the Cu- $\text{A}\beta$ formation and the subsequent development of hydrogen peroxide and free radicals (Cuajungco et al., 2000). However, an excess of zinc is largely toxic in the brain with thus the use of chelating agents in order to avoid the excess. Among chelating agents, TPEN and Clioquinol were used. In “*in vivo*” experimental models of airway inflammation, TPEN attenuates the upregulation of TNF α , IL-13 and IL-4 (Fukuyama et al., 2011). In “*in vitro*” monocytes from adult human stimulated with LPS (mimicking a condition of inflammation), zinc ($100 \mu\text{M}$) provokes a strong induction of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6) blocked by TPEN ($10\text{--}50 \mu\text{M}$) via PKC and NF- κB inhibition (Haase et al., 2008). The same decrements in IL-6 and TNF- α production is also observed in old lymphocytes by EDTA treatment after exposure to high doses of zinc ($20\text{--}100 \mu\text{M}$) (Giacconi et al., 2011). In other “*in vitro*” models, TPEN limits the neuronal cell death provoked by the zinc release by NO in neurons, via p38MAPK kinase (Bossy-Wetzel et al., 2004). The Clioquinol is considered a compound that acts as a zinc, copper, and iron chelators and used as a potential therapeutic strategy for AD because the three metals are involved in the deposition and stabilization of amyloid plaques. Clioquinol can dissolve amyloid deposits by preventing metal- $\text{A}\beta$ interactions (Bush, 2000). In transgenic mice with Alzheimer symptoms and high $\text{A}\beta$ peptide levels, oral Clioquinol treatment for 9 weeks has led to a 49% decrease in brain $\text{A}\beta$ deposition and an improvement of symptoms (Cherny et al., 1999). However, some authors have reported that Clioquinol inhibits the 20S proteasome function by acting through Cu-dependent and Cu-independent mechanisms (Mao and Schimmer, 2008), causing cell death because of the intracellular accumulation of misfolded proteins with thus a possible deleterious role. This dangerous effect is dependent by inflammatory genetic susceptibility or by the doses used, as shown in different animal models where the toxic effect of Clioquinol varies from specie to specie. Anyway, low doses of Clioquinol ($20\text{--}30 \text{ mg/kg/day}$) provoke limited toxic effects at least

in humans (Bareggi and Cornelli, 2010). However, a double-blind, placebo-controlled study of Clioquinol in a population of patients with mild-to-moderate and severe AD (treated with 375 mg twice a day for 36 weeks) found that plasma $\text{A}\beta_{42}$ levels decreased after 24 weeks with an improvement of cognitive performances (Ritchie et al., 2003). Clioquinol, as second generation of the compound (named PTB2), in AD patients leads to a significant reduction of $\text{A}\beta_{42}$ concentrations and improves the cognitive state (Lannfelt et al., 2008; Faux et al., 2010) as well as a better synaptic plasticity in Tg 2576 mice (Adlard et al., 2011). Other clinical studies report instead that Clioquinol did not have any significant effect on cognition and disease progression (Sampson et al., 2008). A promising zinc chelator is 1,10 phenanthroline because reducing $\text{A}\beta$ production in APP overexpressing cells (Caragounis et al., 2007). However, the role of 1,10 phenanthroline is very intriguing in cancer, where an excess of zinc might favour the proliferation of cancer cells (Wang et al., 2006). Zinc chelator 1,10 phenanthroline reduces the concentration of intracellular zinc in HT-29 colon cancer cells by inhibiting Krüppel-like factor 4 (KLF4) and cyclin D1 gene expressions, thereby promoting the arrest of cancer cell proliferation (Huesca et al., 2009). The main effects of zinc chelators are reported in Table 4. Anyway, the zinc state and the individual genetic background (mainly referred to inflammatory genes) play key roles in order to visualize subjects or patients to be treated with zinc supplementation or zinc chelators in order to effectively discern the existing discrepancies in zinc supplementation or zinc chelation.

5. Copper and copper-gene interactions

Copper (Cu) is an essential component of countless enzymes and other proteins and is critical for numerous reactions necessary for growth and development in humans and animals (Olivares and Uauy, 1996). The most bioavailable source of copper is in meat and the major storage is in the liver primarily bound to Metallothioneins (MT) (Hartmann et al., 1993). A blood protein, ceruloplasmin (Cp), synthesized by the liver, contains several molecules of copper and is one marker of body copper status because designed, together with albumin, to the transport of the copper within the cells, organs and tissues (Harvey et al., 2009). A recent adult human dietary recommendation for copper (estimated safe and adequate dietary intake) was set at between 1.5 and 3.0 mg Cu/day (Milne, 1998). Changes in the absorption of copper at different ages have indirectly been assessed. Plasma concentrations of copper increase steadily from childhood to old age (Madarić et al., 1994). The changes in circulating copper have been attributed to a decline of biliary secretion, as a component of the regulation of copper absorption, rather than to an increasingly efficient gastrointestinal absorption capacity throughout life. Therefore, copper absorption in ageing does not seem related to intestinal absorption but rather to copper content in the diet, especially milk and glucose, which favour copper absorption in the intestinal lumen, whereas fructose diminishes copper absorption (Wapnir, 1998). Since milk is a preferred beverage in the elderly (Miura et al., 2011), increased copper in old age by milk intake can be justified. However, the absorption of copper is linked to the presence of zinc and iron, which may compete with copper for the binding, as cofactors, with proteins and enzymes in order to confer them the biological activity.

Despite of the presence of this competition, copper serves as a cofactor for many proteins involved in a variety of biological reactions, such as respiration (cytochrome C oxidase), free radical eradication (superoxide dismutase), connective tissue formation (lysyl oxidase), neurological development (dopamine β -hydroxylase), and iron homeostasis (ceruloplasmin) (Prohaska and Gybina, 2004). However, excessive copper is toxic or even lethal for the cells because participates in generation of ROS through

Table 5

Main encoded proteins affected by copper-gene interactions.

Encoded proteins	Function
MT (Metallothionein)	Antioxidant and copper storage
DMT1	Copper transporter
hCTR1	High-affinity copper transporter
HAH1 (human ATOX-1 homologous)	Copper chaperone for ATP7A/B, copper homeostasis and antioxidant defence
ATP7A	Copper transporter in trans-golgi network
ATP7B	Copper transporter in trans-golgi network
CCS	Copper chaperone for SOD1
SOD-1	Intracellular cuproenzyme involved in free radical detoxification
SOD-3	Extracellular cuproenzyme involved in free radical detoxification
Dopamine β -hydroxylase	Catecholamine production
Thiol oxidase	Disulfide bond formation
Thyrosinase	Melanin production
COX17	Copper chaperone for cytochrome c oxidase
SCO1/SCO2	Copper binding protein
COX (CCO) cytochrome c oxidase	Respiratory electron transport chain
Ceruloplasmin	Copper and iron homeostasis, antioxidant
Lysyl oxidase	Cross-linking collagen
Hephaestin	Cuproenzymes enterocyte ferroxidase
Albumin	Copper transporter
Transcuprein	Copper transporter
Clotting Factor V	Blood clotting
Clotting Factor VIII	Blood clotting
IL-2	Immune function

Key references: Ridge et al., 2008; González et al., 2008; Maret, 2006; Hopkins and Failla, 1999; Kirchman and Botta, 2007; Bonham et al., 2002.

Fenton chemistry and in the direct oxidation of lipids, proteins and DNA (Linder and Hazegh-Azam, 1996). Consequently, intracellular copper content is maintained by evolutionarily conserved cellular transport systems that regulate uptake, export, and intracellular compartmentalization with a balance between copper necessity and toxicity (Ridge et al., 2008). Under this profile, some genes affect copper homeostasis and vice versa (Table 5). The copper-gene interactions affect the coding of proteins involved in delivering copper to various cellular targets, such as copper transporters (CTR and DMT-1) and copper chaperones (CCS, COX17, Atox-1, HAH1) (see review González et al., 2008). HAH1 transfers copper to ATP7A/B (identified as P-type ATPases) located in the trans-Golgi network with the specific function to pump copper to copper-dependent enzyme (lysyl oxidase) involved in elastic and collagen crosslinking and in maintaining cellular copper within safe limits (Petrus et al., 1996). Mutations in the ATP7A/B genes are responsible of Menkes or Wilson diseases. When ATP7B is mutated, as in Wilson's disease, Cp levels decrease and copper accumulates in the liver (Mercer, 2001). This finding suggests the crucial role played by ATP7B in copper homeostasis, including copper efflux in presence of copper excess through copper chaperone Atox1 interaction, which is in turn also involved in Cp synthesis (Culotta et al., 1999). ATP7A interacts with Atox1 in also providing copper for secretory cuproenzymes, such as peptidylglycine α -amidating monooxygenase (PAM) (El Meskini et al., 2003), which is relevant for neuropeptide maturations. Therefore, Atox1 is a key copper chaperone for both copper homeostasis and Cp synthesis. Atox1 gene deletion provokes mortality (Hamza et al., 2001), reduced levels of lysyl oxidase (Prohaska and Gybina, 2004) and the neurons are more exposed to oxidative stress (Kelner et al., 2000).

Therefore, a copper alteration in the diet impugns the gene expression and function of these relevant copper chaperones with subsequent altered intracellular copper homeostasis that may also affect the Cp synthesis. As a result, defects in copper distribution as well as in iron metabolism may occur, because Cp acts also as a ferroxidase converting Fe^{2+} to Fe^{3+} and stimulates Fe efflux (Osaki and Johnson, 1969). Thus, the synthesis of Cp by dietary copper is crucial not only for copper body distribution but also for iron metabolism, as shown by the presence of two Cp polymorphisms with an aminoacid change, a C $\rightarrow$ T at nt 1099 (R367C) in exon 6 and a C $\rightarrow$ T at nt 1652 (T551I) in exon 9 in iron overload individuals (Lee

et al., 2001). Moreover, pro inflammatory cytokines (IL-1, IL-6, TNF- α) regulates Cp synthesis and secretion by the liver, with thus a role of Cp in inflammation in scavenging oxygen radicals (Linder and Hazegh-Azam, 1996). Since chronic inflammation occurs in ageing, increased Cp levels in ageing and in some age related diseases, such as atherosclerosis or Alzheimer disease (AD), could be a good inflammatory marker. While for atherosclerosis, the role of copper and Cp are well defined because implicated in the formation of oxidized LDL (Mukhopadhyay et al., 1997), contradictory data exist on the role played by copper in AD pathogenesis. Some authors report that copper enhances the accumulation in the brain of β -Amyloid (Multhaup et al., 1996); other authors instead report that high copper reduces β -Amyloid plaque formation (Phinney et al., 2003). Anyway, high copper may be deleterious because affecting the gene expression of copper chaperones and Cp and influencing the copper-iron interactions with subsequent enhanced inflammation and increased incidence of some age-related inflammatory pathologies (atherosclerosis, AD, diabetes type II) (Kim et al., 2002). Conversely, a paucity of data exists on the effect of copper and the copper-gene interactions in ageing. However, MT is crucial on the role played by copper in ageing, because MT is also a copper chaperone (Coyle et al., 2002), whose function is also to regulate either the copper intracellular availability (Maret, 2006) or the storage of copper in the liver during copper deficiency (Suzuki et al., 2002) and to keep within safe limits intracellular copper in the presence of an excess of copper (Tapia et al., 2004). Therefore, MT synthesis and production as well as MT polymorphisms become relevant during inflammation in order to act as copper reserve in presence of low copper intake. In this context, some novel +647A/C and +1245A/G MT1A polymorphisms show higher copper levels and enhanced SOD in cardiovascular diseases than healthy old subjects (Giacconi et al., 2008a), suggesting a role of MT in transferring copper to SOD for the antioxidant defense against ROS. However, copper deficiency might exist and to be also relevant in ageing taking into account that copper deficiency affects some aspects of the inflammatory/immune response (Munoz et al., 2007), which is in turn impaired in ageing (Mocchegiani et al., 2006). A low intake of copper (0.38 mg/day) in adult humans significantly impairs IL-2 gene expression (Hopkins and Failla, 1999), with a clear evidence of copper-IL-2 gene interaction, with thus a possible link with the impaired innate immunity in ageing. This last finding suggests

the relevance of dietary copper in ageing because affecting some genes related to inflammatory/immune response, despite sensitive biomarkers are missing to assess marginal or moderate copper deficiency in ageing. The metal-speciation analysis may help to discern this last point (see below Section 12).

6. Copper supplementation or copper chelation? Current problems

The necessity of copper for human health derives from its involvement in myriad biological processes, including iron metabolism, antioxidant defense, neuropeptide synthesis and immune functions, but copper may be also potentially toxic when the intake levels are too high (Brewer, 2008) due to its participation, like iron, in the Fenton-type redox reaction provoking oxidative damage (Linder and Hazegh-Azam, 1996). Therefore, it is important to distinguish two levels of interventions: the first one as copper supplementation in the case of copper deficiency; the second one as copper chelation in the case of copper excess. This subdivision can seem obvious, but the matter is not so simple because it is very difficult to find a precise biomarker for the copper status and, consequently, the choice for copper supplementation or chelation is somewhat problematic. The difficulty especially arises when the copper status has to be determined taking into account that neither plasma Cu nor plasma cuproenzymes reflect Cu status, which is instead estimated by intake and exposure assessment. The latter are subject to the error inherent to all dietary intake studies and, while suitable for the assessment of macronutrient intake, may not be enough precise to estimate intake of a single dietary micronutrient. This assumption is especially valid for copper due to its content in the drinking water that can vary depending by local changes in the chemical water properties (water hardness and pH) (Danzeisen et al., 2007). As a consequence, a large error of dietary Cu intake assessment may occur. Thus, regulators and public health professionals adopt a predominant conservative approach in Cu-exposure regulation. This approach may not be, however, suitable for an essential trace metal, since a low intake of Cu is dangerous as a too-high intake. The situation is then worsened by the lack of precise Cu biochemical biomarker/s, despite plasma copper, ceruloplasmin (Cp) and Cu/Zn superoxide dismutase routinely are assayed in human studies (Harvey et al., 2009). Therefore, a marginal copper deficiency is difficult to diagnose with respect to severe copper deficiency, which is instead present in various pathological conditions (see review Danks, 1988), which some of them are also related to the aging process, such as macular degeneration, osteoporosis, myocardial disease (Klevay, 2000). Conversely, copper toxicity as a result of a dietary excess generally is not considered to be a widespread health concern, probably as a result of the homeostatic mechanisms controlling copper absorption and excretion (Turnlund et al., 2005). However, copper overload has been documented in various pathological conditions usually related to the aging process, such as atherosclerosis, diabetes type II, and Alzheimer disease (AD), despite in AD the data are strongly controversial reporting also copper deficiency (Brewer, 2007). From this complex picture on the copper status in the body, many studies on the effect of Cu upon the immune response, oxidative stress and bone metabolism, and the subsequent copper supplementation or chelation, were conducted in animals and in adult humans with low or excessive copper content in the diet. In order to study the effect of copper in ageing, the budding yeast *Saccharomyces cerevisiae* was used as a model for cellular aging (Kirchman and Botta, 2007). The majority of these studies was conducted testing plasma copper or cuproenzymes or Cp in order to establish the copper status.

7. Copper supplementation by copper deficiency

Dietary copper deficiency affects both innate and acquired immunity (Munoz et al., 2007). Cu-deficient animals and humans display decreased number of circulating neutrophils, a condition termed neutropenia (Koller et al., 1987), reduced bactericidal macrophage functions coupled with a great susceptibility to infections, shorter survival (Percival, 1995) and decreased NK cell cytotoxicity (Koller et al., 1987). Since IL-2 is crucial for T cell proliferation and NK cell cytotoxicity, Cu treatment (24 μ M), affecting directly the transcription of IL-2 gene (Hopkins and Failla, 1997), restores the reduced secretion and activity of IL-2 by activated splenocytes in Cu-deficient rodents with no modifications of Fe and Zn levels (Bala and Failla, 1992). A similar recovery also occurs for other impaired immunological functions (burst neutrophil activity, blastogenic response to T cell mitogens, balance of Th1/Th2 paradigm), with a mechanism involving NF- κ B transcription factor (Bonham et al., 2002). Of interest, it is the recovery of Th1/Th2 paradigm with a normalization of Th2 cytokines and subsequent improved inflammatory/immune response (Bonham et al., 2002).

Recently, taking into account that copper deficiency is associated with cardiomyopathy (Klevay, 2000), Cu supplement (Cu-histidine) therapy, in young patients with SCO2 mutation and COX deficiency, can reverse hypertrophic cardiomyopathy with normalization of ECG signs and blood pressure (Freisinger et al., 2004). Dietary supplement with various micronutrients (for Cu = 1.2 mg/day for 9 months) in old population with chronic heart failure increases left ventricle ejection and decreases left ventricle volume along with improvement of the quality of life (Witte et al., 2005). The beneficial effect of Cu may occur via increased gene expressions of SOD and VEGF (Jiang et al., 2007), suggesting a role of Cu in angiogenesis, whose reduction is involved in endothelial cell senescence (Erusalimsky and Kurz, 2006).

However, few studies exist in animals and humans on the role played by Cu in ageing. The effect of copper supplementation on oxidative stress and life span was assayed in superoxide dismutase mutants *Saccharomyces cerevisiae*. Mutants with a deletion of the SOD1 and SOD2 genes have a very short life span. Copper supplementation increased the life span of the SOD1 and SOD2 mutants, indicating that copper supplementation increases longevity by reducing or removing the superoxide production, via increased gene expression of metallothionein Cup1 (Kirchman and Botta, 2007). In humans, the copper status, tested by plasma copper or Cp or cuproenzymes, is strictly dependent by individual dietary habits and healthy status. In particular, both excessive Zinc or Fe intake can induce copper deficiency because interfering with copper absorption in enterocytes (Underwood, 1981). However, in general, the plasma copper does not have any substantial changes in condition of healthy status during the whole life of an individual including old age, where a copper deficiency is at subclinical level (Harvey et al., 2009). In condition of specific intestinal malabsorption (such as celiac disease, bowel syndrome, long-term parenteral nutrition) or in bone abnormalities or in well genetically determined disease (Menkes' disease), copper deficiency is severe with dysfunctions on immune response, antioxidant activity and bone metabolism (Danks, 1988). In the latter, the copper deficiency is linked to the reduced activity of enzyme lysyl oxidase with subsequent appearance of osteoporosis, which is a pathology characteristic of old postmenopausal women (Lowe et al., 2002). Copper supplementation in old post-menopausal women induces a better bone mineral density (Saltman and Strause, 1993) and an improved clinical outcome of elderly patients with femoral neck fracture (Delmi et al., 1990).

A relevant aspect is the presence of copper and zinc in the photoreceptors and the retinal pigment epithelium (RPE) of the human eye (Ugarte and Osborne, 2001). Several lines of evidence suggest

Table 6A

Effects of copper supplementation in copper deficiency.

Condition	Type of supplementation	Target	Effect
Neutropenia (animal model) (Bala and Failla, 1992)	6 µg of Cu in the diet for 5 weeks	Macrophage functions Neutrophils activity IL-2 mRNA NK cytotoxic activity Th1/Th2 balance T-cell proliferation	↑ ↑ ↑ ↑ Normalization ↑
Cardiomyopathy (human) (Freisinger et al., 2004)	s.c. injections of Cu-histidine (30 µg/kg b.w./day/60 days)	Blood pressure ECG	Normalization normalization
Ageing (animal model) (Kirchman and Botta, 2007)	62 µl of CuSO ₄ in the medium + glycerol	Lifespan in <i>Saccharomyces cerevisiae</i>	↑
Menkes' disease (human) (Kreuder et al., 1993)	s.c. injections of Cu-histidine (65 µg/kg/day/120 days)	IL-2 NK cytotoxic activity SOD	↑ ↑ ↑
Macular degeneration (human) (AREDS study, 2001)	80 mg zinc oxide + 2 mg of cupric oxide/day for 4–11 years	Apoptosis of retinal pigment epithelium SOD	↓ ↑
Osteogenesis in postmenopausal women (human) (Saltman and Strause, 1993)	3 mg CuSO ₄ /day for 2 months	Metallothionein Bone mineral density	↑ ↑
Alzheimer (animal model) (Bayer et al., 2003)	25 g/L of CuSO ₄ in H ₂ O for 3 months	SOD Aβ accumulation	↑ ↓

↑, Increase ↓, decrease.

that intracellular zinc and copper depletion induces apoptosis of RPE and retinal cells and impairs intracellular metal homeostasis by altering the activity of RPE antioxidant metalloenzymes and redox-cycling reactions. As a consequence, increased oxidative stress and subsequent RPE cell damage and photoreceptor degeneration occur with the appearance of age-related macular degeneration (AMD) (Erie et al., 2009). A reduced zinc pool in RPE may be ascribed to reduced action of Metallothionein (Nicolas et al., 1996), whereas reduced copper-adenosine triphosphates (ATPases) has been proposed (Krajacic et al., 2006). Combined oral supplementation of copper plus zinc reduces the risk of progression of AMD in old patients due to best MT homeostasis and increased SOD (AREDS study, 2001). A picture of the copper supplementation in aging and in some age-related degenerative diseases is reported in Table 6A.

8. Copper chelation by copper excess

With regard to high copper, the presence of chronic inflammation plays a pivotal role taking into account that pro-inflammatory cytokines affects the synthesis and production of Cp (Linder and Hazeogh-Azam, 1996), which in turn is under the control of copper status, with thus a strict interrelationship among inflammation, Cp and copper plasma levels. Such a link is evident in some age-related inflammatory pathologies, such as atherosclerosis and diabetes type II (Ford, 2000; Klipstein-Grobusch et al., 1999). On the other hand, elevated copper levels oxidize LDL triggering the atherogenic process (Stadler et al., 2004). These studies show, on one hand that high copper is toxic because generating ROS, on the other hand they suggest that a clinical trial by copper chelation may be of benefit in atherosclerosis. However, to date no clinical trial of copper chelation in atherosclerosis has been carried out. Promising data are obtained in animal models and in type II diabetes using copper chelator as well. Cooper et al. (2004) have shown that in diabetic rats and in type II diabetes the copper chelator *trientine* alleviated the heart failure, improved the cardiomyocyte structure, and reversed elevations in left ventricular collagen and beta-1 integrin without lowering blood glucose. In Wilson disease, the copper-lowering agent tetrathiomolybdate (TM), provokes a greater degree of copper depletion than other drugs in attenuating neurological symptoms (Brewer et al., 2009) (Table 6B).

Of particular interest is the role played by copper in Alzheimer disease (AD) with however contradictory data and different

suggestions (see review Quinn et al., 2009). In this context, AD patients show high free copper correlated positively with peroxide, which can be restored by the copper chelator *Penicillamine* (Squitti et al., 2002). Copper added to the drinking water in a rabbit model of AD greatly enhances the ε3/3 accumulation of β-Amyloid in the brain (Sparks, 2004), which can be inhibited by *cloquinol* treatment (Cherny et al., 2001). Another lipophilic zinc/copper/iron chelator *DP-109* reduces the burden of amyloid plaques and the degree of cerebral amyloid angiopathy in brains from aged hAβPP-transgenic Tg2576 mice (Lee et al., 2004). However, a significant controversy exists over whether an excess of copper is involved in AD pathogenesis. Indeed, accumulating data suggest that increased copper in the brain, due to amplification of a copper transporter (ATPase7b), reduces β-Amyloid accumulation (Phinney et al., 2003). Moreover, copper supplementation in AD mouse model (APP23 transgenic mice) lowered β-Amyloid production, restored SOD, and increased longevity (Bayer et al., 2003). In human AD, the cognitive decline is positively correlated with low copper plasma levels (Pajonk et al., 2005), suggesting thus a possible copper supplementation. Therefore, the evidence is conflicting as to whether too much or too little copper is involved in AD pathogenesis. A significant help to discern this problem may come by the analysis of Apolipoprotein E-4 (ApoE4) cysteine allele that may be involved in copper binding and related to diminished antioxidant effects of E-4 allele (Miyata and Smith, 1996). In AD, the presence of ApoE allele ε4 is associated with increased vulnerability of the brain to the effects of the disease, whereas the presence of the ApoE genotype ε3/3 appears to provide moderate neuroprotection (Alberts et al., 1995). These findings are very attractive because of the possible association between these genotypes and copper status in AD, with thus a very useful help to discern the role played by copper in this pathology with subsequent possible copper supplementation or chelation based on the specific genetic background of the patients. This last concept, as nutrigenomic or nutrigenetic approach, is valid for all the conditions in which copper homeostasis is involved with subsequent major beneficial effects of the copper therapy (supplementation or chelation).

9. Iron and iron-gene interactions

Iron is an essential growth factor for the proliferation and differentiation of all living cells, being centrally involved in: (a) oxygen

Table 6B

Effects of copper chelation in copper overload conditions.

Condition	Type of chelator	Target	Effect
Type II diabetes (human) (Cooper et al., 2004)	Trientine (1.2 g/day/6 months)	Left ventricular hypertrophy (LVH)	Normalization
Diabetes mellitus (animal model) (Cooper et al., 2004)	Trientine (11 mg/day/7 weeks)	Cardiomyocyte structure	Improved
Wilson's disease (human) (Brewer et al., 2009)	Tetrathiomolybdate (120 mg/day/8 weeks)	Beta1 integrin	↓
Alzheimer (human) (Squitti et al., 2002)	Penicillamine (600 mg/day for 24 weeks)	Ceruloplasmin and copper levels	↓
		SOD	↑
		Aβ accumulation	↓
		Cognitive status	↑
Alzheimer (animal model) (Cherny et al., 2001; Lee et al., 2004)	Clioquinol (Oral treatment of 30 mg/kg/day for 9 weeks)	Aβ accumulation	↓
		Survival	↑
	DP-109 (0.1 ml/10 g b. w. for 3 months)	Aβ accumulation	↓

↑, Increase; ↓, decrease.

transport by hemoglobin and myoglobin; (b) electron transport during mitochondrial respiration; (c) the regulation of transcription or as a central component of ribonucleotide reductase or as modulator in catalyzing the process of hydroxyl radical formations (Fenton reaction) (Wang and Pantopoulos, 2011). Such radicals can affect the binding affinity of critical transcription factors, such as hypoxia inducible factor-1 or NF-κB and therefore the transcription of stress-inducible genes (Rosen et al., 1995).

The mismanagement of iron in cellular systems can lead to the toxic accumulation of iron in organ systems, such as the liver and brain, with the production of free radicals with subsequent oxidative stress, cellular damage and eventual cell death via apoptotic signalling (Crichton et al., 2002). Therefore, the absorption, transport, utilization and storage of iron in the body is pivotal for the correct functioning of many body homeostatic mechanisms in order to detect the possible presence of iron deficiency or iron overload or other abnormalities of iron metabolism. These aspects acquire particular relevance during ageing because iron deficiency and iron overload may exist (Lynch et al., 1982), taking into account that changes in iron metabolism occur with advancing age, particularly in women (Martin et al., 1998).

However, such a subdivision between iron deficiency and iron overload might be difficult to estimate because the iron status is dependent by a balance between the iron intake (through the consumption of red meat, poultry or fish) and the iron stores, with a further complication in elderly due to the association between moderate increase in body iron stores and risk for the appearance of some age related diseases, including heart failure and neurodegeneration (Salonen et al., 1992). On the other hand, many of the effects by iron in ageing are not due to the quantity of iron dietary intake but rather to abnormal cellular iron metabolism, trafficking or storage (Levenson and Tassabehji, 2004). Dietary iron is absorbed predominately in the duodenum and proximal jejunum as either heme or non-heme associated iron. The ferrireductase DcytB, a heme-containing enzyme at the apical surface of the enterocyte, uses electrons from NADPH to convert Fe³⁺ into the more readily absorbable form of iron, Fe²⁺ (McKie et al., 2002). Fe²⁺ is then transported across the apical membrane by the transmembrane transporter DMT1, also known as DCT1 or Nramp2, into the cells and stored in mitochondria (Canonne-Hergaux et al., 1999). During ageing, the iron absorption is defective leading to iron deficiency (Garry et al., 1983). However, despite the required quota of iron intake is low (8 mg/day) with respect to young-adults (18 mg/day) (RDA), iron overload may also occur in ageing, especially when iron transporter proteins are altered.

In elderly, an iron deficiency may lead to anaemia (named: Iron Deficiency Anaemia (IDA)) that is different from anaemia caused by the presence of a chronic disease (named: Anaemia of Chronic Disease (ACD)). However, in both conditions the main markers of the iron status, (i.e. ferritin, transferrin, serum transferrin receptor, erythrocyte protoporphyrin and total iron binding capacity) are

altered. In particular, serum ferritin decreases during the first stage of iron deficiency as iron stores are depleted. The optimal cutoff for the detection of iron deficiency in the aged is a serum ferritin concentration ≤45 µg/L (Guyatt et al., 1990). Although ferritin may be a good marker to detect the iron status, the iron-gene interactions may be the added value. Iron affects many genes related both to iron metabolism and inflammatory/immune response on target cells with different responses depending on specific polymorphisms or mutations of these genes. Moreover, iron affects the gene transporters Nramp1 and Nramp2 and a variety of genes related to cell functions, cell growth and metabolism, such as ribonucleotide reductase, cytochrome C, pyruvate dehydrogenase, myoglobin, Rb, p21, cdk2, cyclins A, D3, E1, myc, iNOS, FasL (Table 7). Many of the proteins involved in iron homeostasis can be regulated at the translational rather than transcriptional level. The mRNAs of ferritin, transferrin receptor, aminolevulinic acid synthetase, ferroportin, m-aconitase, and Nramp2 are regulated by an iron responsive element (IRE) on the mRNA, which binds proteins to regulate translation (Umbreit, 2005).

Mutation in the IRE box of the ferritin H gene (FTH1) leads to increased iron storage in the liver with subsequent iron overload resulting as toxic (Hayashi et al., 2006). Ferritin is regulated at multiple levels in response to different stimuli. At translational level, ferritin is regulated by IRPs (Iron Regulatory Proteins), which bind to IRE in the 5'-UTR (5'-untranslated region) of ferritin and repress its translation (Rouault, 2002). IRPs also bind to IREs in the 3'-UTR of TfR1 (transferrin receptor-1), where they stabilize TfR mRNA (Brittenham et al., 2000). Since iron decreases the activity of IRP1 and destabilizes IRP2, the net effect of excess iron is to increase iron storage via ferritin and to decrease iron transport by TfR1. Conversely, the effects of iron restriction are in decreased ferritin and increased TfR1 expression (Hentze and Kühn, 1996). Ferritin and TfR1 are thus key components of the intrinsic homeostatic mechanism used by the cells to restore iron balance when confronted with excess or insufficient iron. On the other hand, transferrin receptor concentrations increase after that iron stores are depleted and ferritin becomes deficient, as it occurs in IDA, but not in ACD (Garry et al., 2000). This finding suggests that serum ferritin remains predictive for iron depletion, but the concentration that determines iron depletion is uncertain even if should be increased in elderly with a value >12 µg/L (Johnson et al., 1994). Anyway, serum ferritin ≤50 µg/L is the best discriminant between IDA and ACD in elderly population (Ioannou et al., 2002).

Other markers for detecting iron metabolism are available. In particular the measure of soluble Transferrin receptor (sTfR) level and the ratio sTfR/log serum ferritin level are promising parameters to distinguish among IDA, IDA with concurrent chronic inflammation or infection, and ACD in elderly population (Joosten, 2004). Indeed, transferrin and its receptors (TfR1 and TfR2) are very important for iron transport, absorption, storage and utilization. They are (i) highly expressed in developing erythrocytes and proliferating

Table 7

Main encoded proteins affected by Iron-gene interactions.

Encoded proteins	Function
HFE	Iron overload and hemochromatosis
β2-Microglobulin	Iron overload related
Ceruloplasmin	Iron and copper transport
Hepcidin	Iron homeostasis
Ferritin-L	Iron storage and transport
Ferritin-H (FTH1)	Iron storage and transport
Transferrin (TfR1/2)	Iron transport, absorption, storage and utilization by tissue and cells
Ferroportin	Iron transport
NRAMP1, NRAMP2	Iron transport
Calreticulin	Iron storage and transport
IRP-1	Iron regulatory
IRP-2	Iron regulatory
Haptoglobin	Iron salvage
Heme oxygenase 1/2	Iron salvage
TNF α	Immune system
Ribonucleotide reductase	DNA synthesis
Cytochrome c oxidase	Respiratory electron transport chain
Pyruvate dehydrogenase	Cellular metabolism, glycolysis
Myoglobin	Specific oxygen-binding protein related to skeletal muscle function
Retinoblastoma (Rb)	Cell cycle control and cell differentiation
Cyclin-dependent kinase inhibitor 1 (p21)	Cell cycle control
Cyclin-dependent kinase 2 (cdk2)	Cell cycle control
Cyclins A, D3, E1	Cell cycle control
Myc	Cell cycle progression, apoptosis and cellular transformation
iNOS	Nitric oxide production
FasL	Apoptosis
Aminolevulinic acid synthetase	Heme biosynthetic pathway
m-aconitase	Mitochondrial function
Iron responsive element (IRE)	Cellular iron homeostasis

Key references: Umbreit, 2005; Hayashi et al., 2006; Brittenham et al., 2000; Zaahl et al., 2004; Tanaka et al., 2010.

cells; (ii) are regulated by IRE-IRP regulatory system (Ponka and Lok, 1999); (iii) and their synthesis is promoted by low intracellular iron level and inhibited by high iron levels (Hentze et al., 1988). In this context, a bulk of data reports the presence of polymorphisms for transferrin and transferrin receptors in normal subjects, in anemia and in iron HFE C282Y homozygous subjects, who display HFE gene mutation, iron overload and hemochromatosis (Adams et al., 2007; Douabin-Gicquel et al., 2001). However, no differences in serum ferritin or transferrin levels among normal, anemic and hemochromatosis subjects are reported (Lee et al., 2001). These findings, while on one hand show the relevance of the iron-gene interactions in determining the nature and the etiology of some iron-related pathologies, on the other hand they do not give any informations on the functional role of these polymorphisms. Especially, they do not give any support on the existence of a precise biomarker for testing the iron status. Thus, further studies are required and the metal speciation analysis may be of support to highlight the iron homeostasis (see below Section 12). Anyway, HFE gene is of interest as possible genetic marker of ageing because a substantial number of old men individuals are heterozygous for the relatively common C282Y mutations with the appearance of increased iron stores (Garry et al., 1997; Steinberg et al., 2001), altered inflammatory/immune response (Wang et al., 2008), increased risk for cardiovascular diseases (Tuomainen et al., 1999; Fleming et al., 1998; Garry et al., 2000) and Alzheimer dementia as well as shorter survival (Bathum et al., 2001; Candore et al., 2003). However, HFE mutations have been also associated with the longevity (Lio et al., 2002; Carru et al., 2003), suggesting that heterozygosity for C282Y may have a higher mortality rate in the younger groups but becomes beneficial in the oldest old (*antagonistic pleiotropy theory of ageing*). Thus, all the findings in HFE gene, together with the findings in MT gene-zinc interaction in ageing (Mocchegiani et al., 2006), are very interesting because suggesting that the longevity is the result of interactions between genetic and environmental factors (especially micronutrients) that in different age may result in successful or unsuccessful ageing. Which

of these interactions (iron or zinc-gene interactions) is the most prominent during ageing in the complex genetic network remains to be established.

However, taking into account that a good inflammatory/immune response plays a key role in achieving the longevity (Mocchegiani et al., 2007), the regulatory gene-iron-immunity network in ageing is pivotal. Indeed, an intriguing aspect is the iron-gene (Nramp1 and Nramp2) interactions related to macrophage function in killing bacteria. A deficiency in these interactions leads to anemia and increased susceptibility to infections as well as increased TNF-α with subsequent chronic inflammation (Bellamy, 1999). Polymorphisms of the iron transporter (Nramp1) in the promoter region of the gene (−237C → T) (Zaahl et al., 2004) influences the gene transcription with a great vulnerability to infections coupled with altered inflammatory/immune responses (Bellamy, 1999). Polymorphisms of Nramp1 (SLC11A1) were also found in Alzheimer dementia, but with a slight association with the disease (Jamieson et al., 2005). Mutation of Nramp2 gene (at nt C1246T) leads to microcytic anemia, increased inflammation, iron overload with no iron recycling (Iolascon et al., 2006). Although no data exists up to date on Nramp1 and Nramp2 polymorphisms in elderly, the role played by these polymorphisms on the inflammation in ageing can be crucial because the altered gene expression of these iron transporters leads to iron stores in various tissues and organs coupled with inflammatory degenerative pathologies (infections, cancer, autoimmune diseases) and very limited redistribution or recycling of iron in the body (Cellier et al., 2007). Therefore, the study of these polymorphisms coupled with a more precise iron status detection in elderly is strongly encouraged.

Anyway, in ageing, the iron stores and the limited iron recycling can be related to the binding between high hepcidin hormonal levels and iron transporter ferroportin, with thus an excessive storage of iron within the cells (Wang and Pantopoulos, 2011). Mitochondria dysfunction can be also involved in iron stores in ageing, because of no transformation, via Fenton reaction, of Fe²⁺ to Fe³⁺ by

m-aconitase enzyme (Ma et al., 2009), with subsequent no redistribution of Fe^{3+} from mitochondria within the body followed by iron stores, oxidative damage, cellular senescence and cell death (Gille and Reichmann, 2011).

Recently, polymorphisms of the transmembrane protease serine 6 (TMPRSS6) gene, an enzyme that promotes iron absorption and recycling via hepcidin, has been found in elderly (Tanaka et al., 2010) as well as polymorphisms in the gene region encoding Iron-Responsive Element-Binding Protein 2 (IREB2) (known as m-aconitase homologue) have been reported in AD (Coon et al., 2006). Thus, these findings suggest the existence of a close relationship between genes and iron metabolism and pin-point the pivotal role of the iron-gene interactions in affecting many body homeostatic mechanisms in ageing. Consequently, a specific and personalized treatment (iron supplementation or iron chelation) in relation to iron deficiency or iron stores, respectively, on the basis of individual genetic background may be carried out.

10. Iron supplementation by iron deficiency

Iron deficiency affects both T cell-mediated and adaptive immunity. Iron deficiency may cause thymic atrophy and decreases the number of peripheral blood T lymphocytes and NK cells, where T-helper and T-suppressor cells are very sensitive to limited iron availability (Santos and Falcao, 1990). A decreased number of naive T-helper (CD4^+ CD45RA^+) and T-cytotoxic (CD8^+ CD73^+) cells in blood from iron-deficient subjects occurs (Munoz et al., 2007), suggesting that iron is also required for the regeneration of new CD4^+ T lymphocytes and maintenance of T cytolytic processes. Of particular interest is the role played by iron deficiency in adaptive immunity. A strong imbalance in Th1/Th2 paradigm occurs with low production of Th1 cytokines (IFN- γ , IL-2) leading to a shift towards Th2 (IL-4) and monocyte cytokine (IL-6) productions with subsequent chronic inflammation and altered inflammatory/immune response (Ekiz et al., 2005). Such a condition (iron deficiency and impaired immune functions) are often associated with anemia that is an usual condition in elderly and its prevalence increases with advancing age. It affects quality of life, cognitive and physical function and is a comorbidity condition that affects other diseases (heart disease, cerebrovascular insufficiency) and also associated with a risk of death (Eisenstaedt et al., 2006). In elderly, many underlying conditions lead to anemia. Other than iron, also folate (folic acid, vitamin B9), vitamin C (acid ascorbic) and vitamin B12 (cobalamin) are deficient in anemia (Izaks et al., 1999; Carmel, 2001). The prevalence of anemia increases after age 60–65 years, and rises sharply after the age of 80 years (Guralnik et al., 2004). Results from the third National Health and Nutrition Examination Survey (NHANES III) indicate that the prevalence of anemia was 11% in community-dwelling men and 10.2% among women ≥ 65 years of age with Hb value < 11 g/dL (NHANES, 2007). Because elderly subjects often have several associated comorbidity conditions and commonly take a variety of medications, some of which may contribute to anemia, the etiology of anemia is frequently difficult to determine even after extensive investigations, including bone marrow examination (Woodman et al., 2005). A significant proportion of elderly anemic subjects (30–50%) are presumed to have multiple causes for their anemia, including malnutrition, intestinal malabsorption and chronic degenerative diseases (Carmel, 2001; Woodman et al., 2005). Taking into account that anemia, other than by iron deficiency, is also due to folate and Vit. B12 deficiencies, the nutrient-deficiency anemia in elderly is generally hyporegenerative and represents the consequence of the older hematopoietic system to replace the peripheral blood loss (Carmel, 2001; Izaks et al., 1999). Hence, iron supplementation (300 mg tablet of ferrous sulphate (60 mg of

elemental iron)) (Umbreit, 2005) or ascorbic acid (Vit. C) has been carried out in elderly anemic subjects (Fleming et al., 1998). The latter is useful because Vit. C increases iron absorption in the duodenum by reducing Fe^{3+} to the more soluble Fe^{2+} with no iron overload (Cook et al., 1991). However, two forms of dietary iron, heme and nonheme, have to be considered for the best iron absorption in the intestinal lumen, because both forms are absorbed with different mechanisms involving other dietary factors. The absorption of heme iron is unaffected by other dietary factors (Hallberg, 1974), whereas the absorption of nonheme iron can be enhanced or inhibited by various dietary components. The major enhancers of nonheme are Vit. C (Hallberg et al., 1986) and meat (Hazell et al., 1978); the major inhibitors are phytate (Reddy et al., 1996), fibers (Simpson et al., 1981), polyphenols (Tuntawiroon et al., 1991) and calcium (Hallberg et al., 1992). From this complex picture of iron absorption, it emerges the complexity of the iron supplementation in elderly, where the status of intestinal absorption, the dietary habits, the healthy status related to the inflammatory condition as well as the ferritin levels have to be considered with a great attention. In particular, the association between ferritin levels and the inflammation plays a pivotal role because increased inflammation and ferritin lead to iron stores with subsequent appearance of degenerative diseases, including cardiovascular disease, diabetes and cancer (Eisenstaedt et al., 2006). Studies in centenarians support this last point of view showing ferritin levels within the normal range (about 114 $\mu\text{g/L}$), limited iron stores (Italian Multicentric Study on Centenarians IMSC, 1998), a low grade of inflammation and reduced appearance of degenerative diseases (Franceschi, 2007). Therefore, the inflammation and ferritin play a pivotal role in iron homeostasis and iron stores for healthy ageing. Under this profile, heme and nonheme iron supplementations were carried out in elderly exclusively in determining the possible set point of ferritin for iron stores (estimated about 60–70 $\mu\text{g/L}$) (Hallberg et al., 1997). Clinical trials in healthy elderly with heme or nonheme iron have shown that the iron stores is strictly dependent by the iron status with no or limited influences on ferritin levels, suggesting that, in presence of a good intestinal absorption, an excessive iron stores does not occur with subsequent best iron redistribution within the body and healthy status as well (Garry et al., 2000; Fleming et al., 1998). Iron supplementation (30 mg Fe/day) in elderly anemic patients (with serum ferritin ≤ 45 $\mu\text{g/L}$) shows a rise plasma ferritin concentration (up to 75 $\mu\text{g/L}$) with a better erythropoiesis without or limited iron stores (Fleming et al., 2002). Clinical trials with Vit. C have been also performed with contradictory results. Some authors found a significant positive correlation between mealtime Vit. C intake and serum ferritin but not total Vit. C intake, suggesting that total daily Vit. C may not be the best indicator of the influence of ascorbic acid on iron stores (Milman et al., 1990). Others report instead no correlation between total Vit. C and serum ferritin (Fleming et al., 1998) with thus a beneficial effect of Vit. C because of no iron store. From this picture, iron supplementation alone or associated with Vit. C in elderly may be useful exclusively in presence of iron deficiency anemia because avoiding the possible iron stores, but not in anemia by chronic disease (ACD). To date, a paucity of data exists on iron supplementation in iron deficiency in relation to some genes. Only one paper reports loss of body hair in mice known as “mask” mice that are iron deficient as a result of a mutation in *Tmprss6* gene coupled with high levels of the hepcidin protein. Iron supplementation (2% carbonyl iron for two weeks) in these mice reverses the iron deficiency and restores hair growth (Du et al., 2008). In humans, the association between *Tmprss6* gene mutation and alopecia remains to be established. However, a close relationship was found among hair loss, iron deficiency and ferritin levels (< 40 $\mu\text{g/L}$) in post-menopausal women affected by alopecia (Deloche et al., 2007), but no iron supplementation was performed. A picture of the effect of various type

Table 8A
Effects of iron supplementation in iron deficiency in elderly.

Condition	Type of supplementation	Target	Effect
IDA (ferritin ≤ 18 $\mu\text{g/L}$) (Umbreit, 2005)	Heme iron polypeptide (60 mg of elemental iron for 6 months)	Haematocrit	↑
		Haemoglobin	↑
		Erythropoiesis	↑
		Transferrin	↑
		Ferritin	↑
IDA (ferritin ≤ 45 $\mu\text{g/L}$) (Fleming et al., 2002)	Heme and nonheme in the meal + Fe (30 mg/day/2 months)	Iron stores	No iron stores
		Reticulocytes	↑
		Haemoglobin	↑
		Erythropoiesis	↑
		Ferritin	↑
IDA (ferritin ≤ 15 $\mu\text{g/L}$) (Fleming et al., 1998)	Nonheme + 36.9 mg of Vit. C for 2 years	Cell-mediated immunity	↑
		Iron stores	No iron stores
		Iron absorption	↑
		Iron stores	No iron stores

↑, Increase; ↓, decrease.

iron supplementation in iron deficiency in elderly is reported in Table 8A.

11. Iron chelation by iron overload

From the concepts expressed above, the possible excessive iron stores and the chronic inflammation in age-related degenerative diseases, provoke oxidative damage and ROS production. Thus, it is necessary to remove from the body the excess of accumulated iron. Some therapeutic strategies have been developed with also the aim to reduce an excess of “free”, “reactive” or “readily available” iron, called “labile iron pool”, because it is toxic promoting further oxidative damage and altering intracellular redox environment (Welch et al., 2002). Phlebotomy is one of the strategies applied to remove excessive iron in patients with one of the forms of primary hemochromatosis and in patients with Peripheral Arterial Disease (PAD) (FeAST study). Although serum ferritin level should be brought to under 10 $\mu\text{g/L}$ and the maintenance phlebotomy is when the ferritin levels are between 80 and 100 $\mu\text{g/L}$ (Bolan et al., 2001), phlebotomy has obtained poor results both in hemochromatosis (Barton and Bottomley, 2000) and in PAD with no effects on the mortality by myocardial infarction or stroke in older patients (Zacharski et al., 2007). Therefore, an alternative strategy is the use of iron chelator *desferrioxamine* (DFO), because inhibiting many parameters related to oxidative stress and inflammation (ROS and pro-inflammatory chemokine productions) in PAD and in Coronary Artery Disease (CAD) (Duffy et al., 2001; Zhang et al., 2010), as well as in reducing atherosclerotic lesions (Minqin et al., 2005) and brain plaque formation in some neurodegenerative age-related diseases (Alzheimer and Parkinson) characterized by excessive iron stores (Brewer, 2007). In this context, a clinical trial with DFO for two years in AD patients shows a slowing in disease progression with however some adverse effects, such as increased inflammation (Crappier McLachlan et al., 1991). In order to avoid this gap in AD treatment, nanoparticles conjugated with iron chelators have been recently developed with encouraging results because reducing the metal load in neural tissue with subsequent attenuation of the oxidative damage (Liu et al., 2010). Another iron chelator (*deferiprone* (DFP)) used in ACD shows poor changes in net body iron stores (Kakhlon et al., 2010). Therefore, iron chelators need further investigations for their use in clinical trials. The effects of the main iron chelators are reported in Table 8B.

Alternatively, the association between “iron management genes” with AD (Zecca et al., 2004) is intriguing, in which mutations of HFE C282Y and H63D genes and the presence of allelic variants of transferrin (subtype C2) increase the risk of AD of 5-fold (Robson et al., 2004). Since the “system iron overload” may be highly variable within the individuals with HFE mutation or C2 polymorphism

(Rossi and Jeffrey, 2004), the inconsistency of clinical trials with iron supplementation or chelators may be related to the presence of these mutations and polymorphisms, with subsequent less efficacy in patients carrying different allelic variants, as it occurs in old subjects and in atherosclerotic patients carrying specific IL-6 allelic variants (Mocchegiani et al., 2008b; Costarelli et al., 2008). Therefore, it is crucial to determine the specific individual genetic background for the best treatment. Moreover, the specific predictors of iron status are unlikely because highly variable and dependent by the individual healthy status and environmental factors (dietary habits). The detection of metal speciation analysis and the individual genetic background may be useful tools to determine the more correct individual therapy in order to maintain the healthy status or to achieve the outcome from the pathology.

12. Speciation of trace elements in human serum

The assessment of copper or zinc status as well as of other essential trace elements, such as iron, is a long term debated but unsolved challenge in nutritional research. Blood serum, plasma and urine are the most studied biological fluids in the research for nutritional biomarkers as they can be easily obtained and preserved in biological banks for further analysis. As reported above, plasma and tissue content of trace elements, especially zinc and copper are significantly affected by stress, disease and inflammation. Therefore, it is very difficult to find non-invasive biomarkers of zinc or copper status, particularly in “non-healthy” subjects and in presence of chronic or acute inflammatory conditions. The findings in rheumatoid arthritis can be very explicative to represent this concept. In fact, decreased plasma zinc concentration and increased plasma copper seem to be significantly correlated with disease severity, but it appears that the disease develops and progresses without being linked to either copper or zinc deficiency conditions (Milanino et al., 1993). Overall, these considerations suggest that misleading conclusions on nutritional deficiency or toxic accumulation of these essential trace elements can be possibly avoided only establishing a pool of biomarkers that provide information on their metabolism, bio-accumulation and form-specific functional role. According with this point of view a reasonable approach for identifying nutritional biomarkers of zinc, copper as well as other essential trace elements is to compare their distribution among specific proteins with their absolute amounts in serum or plasma. In each biological fluid, specific metal containing proteins are present that are involved in transport processes, acute phase reactions or in the protection of cells against oxidative damage. Taking into account that several homeostatic mechanisms regulating these proteins are well established, a precise quantification of essential trace elements bound to different biomolecules (commonly termed speciation analysis) – at

Table 8B
Effects of iron chelation in iron overload conditions.

Condition	Type of chelator	Target	Effect
Hemochromatosis (human) (Bolan et al., 2001)	Phlebotomy	Lipid peroxidation	↓
PAD (human) (Zacharski et al., 2007)		Ferritin	↓
ApoE ^{−/−} mice (animal model) (Zhang et al., 2010)	DFO i. p. injections, 100 mg/kg b.w/day/14 days	ROS production	↓
Cholesterol-fed rabbits (animal model) (Minqin et al., 2005)	s. c. injections of 72 mg/kg/day/5 days	TNF-α	↓
CAD (human) (Duffy et al., 2001)	Vein infusion of 500 mg over 1 h at 8.3 mg/min	MCP-1	↓
		APP	↓
		NO	↑
Alzheimer (human) (Liu et al., 2010)	Nanoparticles conjugated to iron chelators: (MHEHP or MAHP or MAPHP)	ROS production	↓
ACD (human) (Kakhlon et al., 2010)	Deferiprone (DFP) 20–30 mg/kg/day for 6 months	Aβ accumulation	↓
		"Labile iron pools" in mitochondria	↓
		m-aconitase	Restoration
		ROS production by mitochondria	↓

↑, Increase; ↓, decrease.

least in one or two biological fluids (i.e. serum and urine) should potentially provide enough information to depict a trace element nutritional status.

Hyphenated techniques based on coupling chromatographic separation techniques, mainly HPLC, with ICP-MS detection are now established as the most realistic and potent analytical tools available for speciation analysis of essential trace elements (Sanz-Medel et al., 2003). This kind of application can be easily carried out by connecting the exit of an analytical or capillary HPLC column to the entrance of the ICP nebulizer and some applications in clinical conditions related to zinc, copper and iron depletion or accumulation have been already carried on (Fig. 1). In order to minimize modification of the original species the choice of chromatographic separation is restricted to anion exchange (AE) and size exclusion (SEC) using aqueous mobile phases buffered at physiological pH. This approach has been recently used to investigate the permeability of the blood-cerebrospinal fluid-barrier for selected metals (Mn, Fe, Cu, Zn, Mg and Ca) by studying their speciation in paired human serum and cerebrospinal fluid samples (Nischwitz et al., 2008). However, SEC separation of element species from biological samples is generally unsatisfactory (Sanz-Medel et al., 2003) and the time required for each chromatographic run is relatively long, taking also into account the necessity to wash the column, after each run, with acidic solutions (Nischwitz et al., 2008) or EDTA (Malavolta et al., 2007). These problems and others related to the requirement of extended optimizations, time consuming elaboration of data and high sample volumes (generally hundreds of microliters) could be overcome using monolithic anion exchange micro columns installed on a tandem HPLC system and simplified systems for calculation of peak areas and concentration (Malavolta et al., 2012). Using AE separation with a slow gradient of ammonium acetate, it is also possible to obtain the fine detection of transferrin isoforms in human serum on a Mono-Q HR 5/5 anion-exchange columns (Arizaga Rodríguez et al., 2005). A similar approach using a linear gradient of ammonium acetate has been used to obtain quantitative speciation of the most important trace elements in human serum (Muñiz et al., 2001; Mestek et al., 2007; Hasegawa et al., 2007). The detection of quantitative and qualitative differences in Fe and Zn speciation of sera from healthy individuals versus patients on hemodialysis suggests that speciation of human serum by SAX-ICP-MS has the potential to be translated into clinical research (Muñiz et al., 2001). A similar approach has been used to obtain an accurate determination of clinical iron status from parameters measured in human serum. Using anion exchange separation with ICP-MS detection and stable isotope labelling, the authors were able to measure transferrin saturation, total iron binding capacity, unsaturated iron-binding capacity and serum iron. The validity of this technique in detecting

iron status was proved by the comparison of these parameters in healthy individuals, iron supplemented subjects and patients with hemochromatosis (del Castillo Busto et al., 2008). Studies using a similar approach to detect the saturation grade of copper and zinc to different serum proteins are currently ongoing in our lab. with the aim to find specific biomarkers for copper and zinc status. A clinical case of a child with high levels of plasma zinc concentration but presenting signs of severe zinc deficiency was investigated by SEC-ICP-MS following administration of an oral dose of ⁶⁷Zn and an intravenous dose of ⁷⁰Zn (Owen et al., 1996). The analysis showed that most of the child endogenous and exogenous (⁶⁷Zn and ⁷⁰Zn) Zn was not associated with normal Zn-binding proteins but seemed to bind to a unconventional high molecular weight protein. This finding helps to explain how a child with high levels of plasma zinc could display symptoms of zinc deficiency. A method that uses immunoaffinity plus SEC with ICP-MS detection has been described for clinical research involving a sensitive and accurate analysis of Cu bound to ceruloplasmin in human serum (Kobayashi et al., 2007). The determination of ceruloplasmin with this method could be useful when disorders of copper metabolism or storage are suspected. The most important clinical application of the ceruloplasmin test is in the diagnosis of Wilson's disease, where typically, concentrations of ceruloplasmin are reduced and concentration of dialyzable copper are increased (Mak and Lam, 2008). Ceruloplasmin assay could be also considered in cases of central nervous system disease of obscure etiology or other conditions that may determine low copper bound to ceruloplasmin such as Menkes' syndrome, overdose of vitamin C, copper deficiency as well as for conditions that determine high levels of Cu bound to ceruloplasmin, such as pregnancy, lymphoma, acute and chronic infections or rheumatoid arthritis. The SEC-ICP-MS methodology has been recently applied to test more accurately copper and ceruloplasmin in Wilson's disease (El Balkhi et al., 2010). Further improvement of this technique by including other essential trace elements other than Cu bound to ceruloplasmin would be desired. However, it would be necessary that ICP-MS detection will be combined with "Time of Flight" or other detection systems (Muñiz et al., 2001; Sanz-Medel et al., 2003) in order to assign all the element species to specific proteins as well as to use speciation results for planning interventions based on modulation of essential trace element homeostasis (Malavolta et al., 2012).

From all these findings on metal speciation analysis, it is evident that the quantification of essential trace element species can be performed with hyphenated ICP-MS technologies and that further improvements of these research tools will be achieved in the next years to translate the results into clinical applications. In particular, these techniques might be useful for the identification of patho-physiological conditions in which zinc, copper, iron

Fig. 1. A representative example of a chromatographic separation of Fe, Cu and Zn species obtained by HPLC-ICP MS and reported clinical applications with the percent (%) of saturation in normal condition and in some pathologies (for details see the text).

and their respective transport proteins (albumin, ceruloplasmin, ferritin and/or transferrin) are involved, including age-related diseases as well as age-related physiological changes with specific range values in the percent (%) of the saturation of the metal with the respective protein in normal condition and in some pathologies (Fig. 1). With regard to how blood plasma levels may reflect tissue

levels for these minerals, work is in progress in our Lab. However, data in the plasma can have a relevant clinical impact.

13. Conclusions and future perspectives

Despite the pivotal role played by zinc, copper and iron for inflammatory/immune response and antioxidant activity in ageing and in some age-related diseases, controversial findings exist on the “real” necessity of micronutrient supplementation in presence of metal-nutrient deficiency or their chelation in presence of metal-nutrient toxicity. The causes may be various and dependent by the dietary habits or by the existence of poor precise biomarkers to diagnose the presence of deficiency or toxicosis, although albumin, ceruloplasmin, ferritin and/or transferrin are the more common proteins used to test in the plasma zinc, copper and iron status, respectively. However, the discrepancies may be more due to the fact that these micronutrients affect many genes related to the inflammation and oxidative stress with subsequent antioxidant enzyme biological functions and cytokine productions, which in turn affect the intestinal absorption and distribution of the micronutrients to various organs and tissues. On the other hand, the relevance of the cytokine production in maintaining a correct Th1/Th2 paradigm, with subsequent satisfactory inflammatory/immune response, has been well documented in ageing in relation to all the three microelements herein reported. Alterations in the absorption or dietary intake of zinc, copper and iron leads to an incorrect inflammatory/immune response, impaired antioxidant activity and unsatisfactory DNA repair to various external noxae with the subsequent appearance of some age-related degenerative diseases (Mocchegiani et al., 2012). Taking into account that pro-inflammatory cytokines also affect the absorption and the distribution of zinc, copper and iron within the body (Prasad, 2000), a vicious circle arises with deleterious or limited beneficial effects of the micronutrients themselves on inflammatory/immune response and antioxidant activity (Fig. 2). Supplementation or chelation have been suggested in relation to deficiency or excess, respectively, of

Fig. 2. Schematic representation of the possible beneficial interactions between dietary micronutrients (Cu, Fe, Zn) intake and the related genes in inducing a satisfactory cellular homeostasis, genomic stability and DNA repair in ageing, where the inflammatory status by pro-inflammatory cytokines and chemokines plays a key role affecting both genomic stability and the absorption and transport of micronutrients. A correct balance of the interrelationship micronutrient-gene-inflammation leads to healthy status and longevity.

the three micronutrients but with contradictory and often unsatisfactory results, especially when encouraging data obtained in “*in vitro*” models are transferred in humans. This gap is mainly due to a great interindividual genetic variability, to the interaction between environmental (dietary habits) and genetic factors and to the unreliability of specific biomarkers for testing zinc, copper, iron status that altogether contribute to the poor successful of the supplementation or chelation. In this contest, polymorphisms of pro-inflammatory cytokines and Metallothioneins are very useful tools to screen old subjects who effectively need micronutrient supplementations with subsequent beneficial effect of the micronutrient itself upon the inflammatory/immune response and antioxidant activity. Such a beneficial effect is the case of zinc and copper where subjects with polymorphisms for IL-6 and MT for zinc and SOD1 and SOD2 for copper can be screened for nutrient supplementation or chelation regardless to the nutritional status and inflammatory condition as well as to the presence of specific pathologies, such as Wilson diseases or Alzheimer type dementia (AD). Such a genetic screening is very useful both in ageing and in age-related diseases taking into account the existence of a strictly relationship between copper and zinc and by the recent discovery showing the Cu/Zn ratio as predictor of disability and mortality in ageing (Malavolta et al., 2010; Mocchegiani et al., 2011). With regard to iron, allelic variants for transferrin of HFE gene mutation are also useful tools for iron overload in AD patients with subsequent beneficial effect of specific chelator DFO. Old subjects and AD patients carrying allelic variants for some pro-inflammatory cytokines (TNF- α) or APOE are more or less susceptible to the nutrients’ absorption and distribution within the body. Therefore, the micronutrient-gene interactions (both as nutrigenomic and nutrigenetic approach) are fundamental to screen subjects for nutrient supplementation or chelation strictly related to the inflammatory and oxidative stress states. However, such a genetic approach may be not enough for a correct individual supplementation or chelation, taking into account that the simple dosage in the plasma or serum of the respective metal-proteins may be misleading for a precise diagnose of the nutrient deficiency or nutrient overload. Indeed, the nutrient may be deficient in the circulation but stocked in various organs and tissues, as it occurs for example for copper and iron in the liver (Hallberg et al., 1997) with subsequent overload of the nutrient and appearance of degenerative diseases. Thus, the protein-metal speciation analysis can be the added value for a precise diagnose of nutrient deficiency or nutrient overload with subsequent a more complete picture for a correct individual nutrient supplementation or chelation in ageing and in age-related diseases with thus the possible achievement of healthy ageing and longevity.

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