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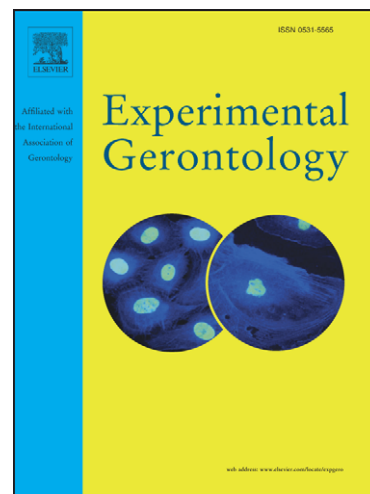
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**Effect of zinc supplementation on plasma IL-6 and MCP-1 production and NK cell function in healthy elderly: interactive influence of +647MT1a and -174IL-6 polymorphic alleles.**

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**Running title:** Zinc, IL-6, MCP-1, NK function in elderly

**Key Words:** zinc, IL-6, MCP-1, NK lytic activity, +647 MT1a polymorphism, -174 IL-6 polymorphism, elderly.

**ABSTRACT**

Pro-inflammatory cytokine response and NK activity are controlled by the availability of zinc ion, whose intra-cellular transport is regulated by metallothioneins.

In order to closely examine the importance of circulating zinc in the modulation of immune response during ageing, in the balance of Th2/Th1 equilibrium and finally in the reversibility of systemic low grade inflammation, we evaluated the changes occurring in plasma IL-6 and MCP-1 concentrations and NK lytic activity in a healthy low grade inflamed elderly population, following zinc-aspartate supplementation. In addition, we aimed to highlight the potential interaction among circulating zinc increments, changes in immunological parameters and +647MT1a and -174IL-6 polymorphic alleles.

39 healthy individuals (60-83 years) from the ZINCAGE cohort (previously typed for +647 MT1a and -174 IL-6 polymorphisms) were supplied with zinc-aspartate. Blood samples collected before and after supplementation underwent basal laboratory determinations (circulating zinc, albumin and C reactive protein) and immunological studies (plasma IL-6 and MCP-1 and NK lytic activity). Zinc supplementation in subjects with low or borderline-normal circulating zinc increased the concentration of this ion and modulated plasmatic IL-6 and MCP-1 as well as NK lytic activity. An interactive effect of polymorphic alleles of MT1a and IL-6 genes on zinc, IL-6, MCP-1 and NK activity was evidenced following supplementation, indicating the genetic background as one of the determinants for identifying groups of subjects that can take advantage of therapeutic intervention.

## INTRODUCTION

Inflammation, ageing and age-associated diseases are conditions possibly characterized by zinc deficiency. Among micronutrients, zinc is one of the most important trace elements required as a catalytic, structural and regulatory ion for the activities of more than 300 enzymes, proteins and transcriptional factors. Therefore, zinc is a key element in many homeostatic responses of the body, including oxidative stress and for many biological functions, including immune efficiency (Fraker and King, 2004; Ibs and Rink, 2003; Rink and Gabriel, 2000).

In particular, zinc deficiency may be related to the decreased functional activity of immune cells, associated with ageing, by reducing cell-mediated adaptive and innate immune responses (Dardenne, 2002; Shankar and Prasad, 1998) as well as adversely affecting the secretion and function of cytokines (Prasad, 2000) and possibly chemokines (Mariani et al., 2006), thus resulting in limited immuno-resistance to infections.

A progressive age-related increase of plasma concentration of some chemokines has been observed in healthy elderly subjects (Inadera et al., 1999; Gerli et al., 2000; Antonelli et al., 2006; Wieczorowska-Tobis et al., 2006). In particular, MCP-1 chemokine levels appear closely associated with age (Deo et al., 2004; Mariani et al., 2006) and elevated in concomitance with low circulating levels of zinc (Mariani et al., 2006). Also acute phase protein increases are described to accompany the ageing process. The finding of these proteins mirrors a chronic low grade inflammatory state (as defined by a 2-4 fold increased level) (Vasto et al., 2007a), determined by different factors such as the chronic antigenic stress (probably the most prominent one). This progressive and prolonged activation of the immune system is evident in the elderly and, along with the genetic characteristics, is possibly related to several diseases of ageing with an inflammatory pathogenesis (such as Atherosclerosis, Type 2 diabetes, Osteoarthritis and Alzheimer's Disease) (Mariani et al., 2007).

Pro-inflammatory cytokine response is largely controlled by the bio-availability of zinc ions and some zinc-dependent transcription factors are involved in the regulation of the gene expression of pro-inflammatory cytokines (e.g. IL-6, TNF- $\alpha$ ) (Mocchegiani et al., 2006; Prasad et al., 2004). Furthermore, zinc-finger proteins are necessary for signal transduction from cytokine receptors to response genes. All these activities are carefully regulated inside the cells by metallothioneins (MT), a group of metal-binding proteins with high affinity for zinc, playing a central role as intracellular zinc transporters. Human MT genes, in turn, are

up-regulated by zinc (Vandeghinste et al., 2000) and some polymorphisms of these genes have been correlated to chronic inflammation and may affect zinc release (MT1a sub-isoform, in particular) (Mocchegiani et al., 2006; Richards et al., 1984). Furthermore, MT induction and expression is under the control of IL-6, a multifunctional cytokine, major component of acute phase response, regulating differentiation and activity of different cell types (including lymphocytes), stress reactions and inflammatory responses (Vasto et al., 2007b).

As a consequence, high circulating levels of IL-6 related to the chronic inflammatory status (and possibly the -174G/C polymorphism in the promoter region of the IL-6 gene), influence abnormally elevated levels of MT that, binding zinc, decrease the availability of this ion for biological processes and immune functions, particularly in ageing (Franceschi et al., 2000, 2005; Olivieri et al., 2006).

In order to closely examine the importance of circulating zinc in the modulation of immune response during ageing, in the balance of Th2/Th1 equilibrium (showing a prevalent Th2 profile in the elderly) and finally in the reversibility of systemic low grade inflammation, we evaluated the changes occurring in plasma IL-6 and MCP-1 (a Th2 chemokine) concentrations and NK cell functional activity in a healthy low grade inflamed elderly population, following zinc-aspartate supplementation. In addition, we aimed to highlight the potential interaction among circulating zinc increments, changes in immunological parameters and +647MT1a and -174IL-6 polymorphic alleles.

## **MATERIALS AND METHODS**

### **Study population**

A sample of 39 healthy individuals, (19 women and 20 men, aged 72.7 years  $\pm$  6.40, mean  $\pm$  SD) (range 60-83 years) was taken from the cohort of the ZINCAGE project and underwent a zinc supplementation trial, that preferentially included carriers of +647 MT1a C+ and -174 IL-6 C- alleles. All subjects undergoing zinc supplementation had concentrations of plasma zinc below 11  $\mu$ mol/L at least at one check-up of two made within one year.

The sample, recruited by Mocchegiani in Italy, Rink in Germany, and Dedoussis in Greece, was supplied with 10 mg/day of zinc-aspartate (Unizink 50, KÖHLER PHARMA Corp., Alsbach-Hähnlein, Germany). Subjects recruited by Jaite in Poland were supplied with an identical form of zinc-aspartate, locally produced (Zincas, Zakład Chemiczno-Farmaceutyczny FARMAPOL, Poznań, Poland). All the subjects received the supplement for 48  $\pm$  2 days.

All participants provided informed consent according to the procedures required by the local Research Ethics Committees, which also approved the study protocol.

Blood samples collected before and after supplementation from overnight fasting subjects underwent basal laboratory determinations. For immunological studies, plasma was separated by centrifugation at 4°C, divided and appropriately stored at -80°C until used for cytokine and chemokine determinations. Cellular pellets were diluted with PBS, stratified on density gradient and centrifuged. Peripheral blood mononuclear cells (PBMC) separated at the interface, resuspended in RPMI 1640 containing FCS and DMSO, were cryopreserved in liquid nitrogen. Laboratory data, plasma, PBMC and DNA samples were kept anonymous.

Briefly, the selection included healthy old people, still living independently (at their own home or in nursing homes) not needing special daily care and not taking any noteworthy drugs (steroids, diuretics, anti-convulsants, anti-depressants, chelating drugs, antibiotics and antimetabolites, or NSADs, in the last two months before the recruitment), nutritional integrators, or vitamin complexes.

#### **Plasma albumin, C-reactive protein and zinc concentrations**

Plasma albumin and C-reactive protein (Crp) concentrations, as indicators of nutritional status and of chronic diseases, respectively, were measured by nephelometry (albumin normal range: 3.5-5.3 g/dl and Crp normal range: <0.3 mg/dl). Concentration of zinc, as a micronutrient involved in the immune response, was determined with a Thermo XII Series induction coupled plasma mass spectrometer (Thermo Electron Corporation, Waltham, MA, USA) as previously reported (Malavolta et al., 2006) (normal range: 10-15 µmol/L). These data were kindly supplied by Moccheggiani's group.

#### **Multiplex bead immunoassay**

IL-6 and MCP-1 concentrations were simultaneously evaluated in duplicate plasma samples, using commercially available multiplex bead-based sandwich immunoassay kits (Bio-Rad Laboratories, Hercules, CA, USA), as previously described (Mariani et al., 2006). Briefly, distinct sets of fluorescently dyed beads (50µl) loaded with capture monoclonal antibodies specific for each cytokine to be tested were incubated with a similar volume of plasma (1:4 diluted) or with standards in a 96-well plate. Subsequently, 25 µl of fluorescent detection antibody mixture were added and then the samples were washed and resuspended in assay buffer.

Low standard curves for each soluble factor were used, ranging from 3,952.5 to 0.24 pg/ml for IL-6 and from 2,280.2 to 0.14 pg/ml, for MCP-1.

The formation of different sandwich immunocomplexes on distinct bead sets was quantified using the Bio-Plex Protein Array System (Bio-Rad Laboratories, Hercules, CA, USA), evaluating the fluorescent signal of a minimum of 100 beads per region (chemokine/cytokine). Data were analysed using the Bio-Plex Manager software version 3.0 (Bio-Rad Laboratories, Hercules, CA, USA). In general, at least six standards were accepted and used to establish standard curves following a five-parameter logistic regression model (5PL).

#### **+ 647 A/C MT1a and -174 G/C IL-6 polymorphisms**

The first polymorphism corresponded to an A/C transition at +647 nt position in the coding region of the human MT1a gene, the second one corresponded to a G/C transition at position -174 in the human IL-6 gene promoter.

Genotyping for each polymorphism was performed by Mocchegiani's group. Amplification conditions were reported elsewhere (Mocchegiani et al., 2006; Olomolaiye et al., 1998).

#### **NK cell lytic activity**

K562 tumor target cells ( $1 \times 10^6$ ) were labelled with 15  $\mu$ M calcein-AM (Molecular Probe, Eugene, Oreg) for 30 min at 37°C and washed 2 times with cold complete medium. Labelled target cells ( $5 \times 10^3/50 \mu$ l) and varying numbers of cryopreserved and thawed effector cells (from  $5 \times 10^6$  to  $5 \times 10^3$  PBMC/100  $\mu$ l) were incubated in triplicate in V-bottom 96-well plates (Effector/Target E/T ratios from 50/1 to 1/1). After incubation at 37°C in 5% CO<sub>2</sub> for 4 h, 75  $\mu$ l of each supernatant were harvested, transferred into new plates and measured by a dual-scanning microplate spectrofluorimeter (Spectramax Gemini - Molecular Devices, Sunnyvale, Calif).

Data were expressed as arbitrary fluorescent units (AFU). Percent lytic activity was calculated according to the formula [(test AFU-spontaneous AFU)/(maximum AFU-spontaneous AFU)] x 100. Spontaneous AFU represents Calcein AM released from target cells in medium alone and maximum AFU is Calcein AM released from target cells lysed in medium plus 2% Triton X100, each measured in at least six replicate wells (Neri et al., 2001).

#### **Statistical analysis**

Concentrations were expressed as  $\mu$ mol/L for zinc, g/dl for albumin, mg/dl for Crp, pg/ml for IL-6 and MCP-1. NK cell function was expressed as % lytic activity. All values were presented as means  $\pm$  SD and/or SEM and as medians and percentiles. Differences between men and women or C- and C+ alleles were evaluated by the Mann-Whitney U-test; differences before and after supplementation were evaluated by Wilcoxon's matched pairs test; differences among sub-groups were analysed by Analysis of variance followed by post-



tests. Correlations among variables were assessed by Pearson's and Spearman correlation coefficients. All reported  $p$  values are based on two-sided tests and were compared with a significance level of 5%. "Statistica" (release 6, StatSoft Inc.) for Windows was used to perform statistical analysis.

## RESULTS

### **Zinc supplementation modulates circulating zinc, IL-6 and MCP-1 and NK cell activity.**

There were no significant differences between men and women regarding age, basal plasma zinc and albumin concentrations (Table 1), therefore they were analysed together. Both men and women had albumin concentrations within the normal range, indicating that none of the examined subjects had clinically evident malnutrition. In addition, all the subjects presented a systemic low grade inflammation but did not suffer from chronic diseases, as indicated by serum C-reactive protein, whose levels never exceeded a 4 fold increase. In particular, 8 subjects were Crp negative, the remaining group presented a median of 0.275 mg/dl before and of 0.31 mg/dl after supplementation (5<sup>th</sup>-95<sup>th</sup> percentiles: 0.1-1.06 mg/dl before and 0.1-1.01 mg/dl after supplementation) without differences between men and women and without modulation by zinc. Also IL-6 and MCP-1 plasma concentrations indicated a systemic low grade inflammatory status, being within a 4 fold increase, compared to normal values (Table 1).

Zinc-aspartate supplementation significantly increased circulating zinc concentrations up to  $11.5 \pm 1.85 \mu\text{mol/L}$  (mean  $\pm$  SD; Wilcoxon matched pairs test,  $p < 0.001$  compared to basal levels) mainly in women, allowing the subjects belonging to the first quartile before supplementation to up-grade to the second quartile after supplementation (not shown). Serum basal concentrations of zinc were directly correlated to the levels reached after supplementation (Pearson's correlation coefficient  $r = 0.536$ ,  $p = 0.0001$ ).

Concomitantly, also IL-6 and MCP-1 plasma concentrations (Table 2) were modified by zinc-aspartate supplementation (up and down-modulated, respectively) as well as NK lytic activity that increased both in men and women (Table 3).

Basal plasma concentrations of IL-6 were proportional to the MCP-1 basal ones (Pearson's correlation coefficient  $r = 0.367$ ,  $p = 0.02$ ), whereas they showed a borderline negative correlation following supplementation (not shown).

In addition, the higher the concentration of IL-6 before supplementation, the lower the increment observed following supplementation was (Pearson's correlation coefficient  $r = -$

0.847,  $p=0.000$ ), suggesting that IL-6 could be modified in the plasma within a limited biological interval.

**Influence of single +647 MT1a and -174 IL-6 polymorphic alleles.**

In the 39 examined subjects, the observed frequencies of MT1a genotypes for +647 A/C polymorphism were compared with the expected frequencies and did not deviate from the Hardy-Weinberg equilibrium (Chi-square = 4.317632,  $df=2$ ,  $p=0.115463$ ), mirroring the whole population from which the examined subjects were taken. On the contrary, IL-6 genotypes for -174 G/C polymorphism significantly deviated from Hardy-Weinberg equilibrium (Chi-square = 7.693303,  $df = 2$ ,  $p=0.021352$ ) for a lower presence of heterozygous subjects in favour of a higher number of the homozygous ones (mainly CC).

AC and CC genotype carriers for +647MT1a polymorphism were grouped and referred to as MT1a C+ (25 subjects), whereas AA genotype carriers were referred to as MT1a C- (14 subjects). Likewise, GC and CC genotype carriers for -174IL-6 polymorphism were grouped and referred to as IL-6 C+ (14 subjects), and GG genotype carriers were referred to as IL-6 C- (25 subjects).

Plasma zinc concentrations, both basal and after supplementation, were lower in C- than C+ MT1a carriers (Figure 1a). However, both C- and C+ carriers tended to increase circulating zinc concentrations after supplementation, albeit not significantly, but maintained their differences as in basal conditions (Figure 1a). As concerning IL-6 polymorphism (Figure 1b), C- and C+ carriers presented similar zinc concentrations in basal conditions, whereas, after zinc supplementation, C+ carriers displayed a borderline significant ( $p=0.06$ ) increase compared to C-, thus undergoing a significant up-regulation compared to their basal concentrations.

In general, subjects displaying relatively high basal concentrations of zinc (e.g. MT1a C+) presented on average a low increment after supplementation (Pearson's correlation coefficient  $r= -0.5764$ ,  $p=0.005$ ).

Basal IL-6 plasma concentrations were slightly lower in MT1a C+ carriers than in C- ones (Figure 1c). Plasma IL-6 increased after zinc supplementation, thus showing an evident up-regulation in MT1a C+ carriers (Figure 1c), but, in general, without differences between C- and C+ carriers.

IL-6 C- and C+ carriers (Figure 1d) showed trends opposite to MT1a polymorphism, with C- carriers presenting lower basal IL-6 concentrations and the most significant increments after supplementation, but, in general, C- and C+ carriers were almost similar.

Basal MCP-1 levels were similar before and after supplementation both in MT1a and IL-6 polymorphic allele carriers (Figure 1e, Figure 1f) and a down regulation of MCP-1 concentrations of about 10% was evident after zinc supplementation.

Lytic activity of NK cells was similar in C+ and C- carriers of both MT1a and IL-6 polymorphisms in basal conditions and was up-regulated by zinc supplementation (Figure 2 a, b, c, d).

The increments (differences between the values reached after and those before supplementation) of circulating zinc, plasma IL-6, plasma MCP-1 and NK lytic activity (at 1/1 E/T ratio, in order to evaluate activity on a per-cell-basis, thus avoiding the amplification effect due to cell recycling) were arranged according to circulating zinc increments. A positive trend (Spearman correlation  $r=0.272$ ,  $p=0.03$ ) of circulating zinc concentrations (Figure 3a) from IL-6 C-, to MT1a C+ and C- carriers and finally to IL-6 C+ was observed (IL-6 C- vs C+,  $p<0.02$ ). In particular, IL-6 C- and MT1aC+ carriers shared low increments of zinc, (below  $1\mu\text{mol/L}$ ), high increments of plasma IL-6 (Figure 3b) (over 70% of basal concentrations;  $p<0.005$  at least, compared to IL-6 C+ and MT1a C-, respectively, not shown), and limited increments of NK lytic activity (Figure 3c). The other two polymorphisms (e.g. MT1a C- and IL-6 C+), parallel to circulating zinc concentrations increments over  $1\mu\text{mol/L}$  (Figure 3a), showed a limited increase of plasma IL-6 (Figure 3b) (lower than 25% of basal concentrations) and more evident increments of NK lytic activity (Figure 3c), (over 80% of basal concentrations).

A general reduction in MCP-1 concentration was observed independently of polymorphic alleles, showing similarities between C- and C+ alleles of both polymorphisms (Figure 3d).

#### **Interactive effects of combined +647 MT1a and -174 IL-6 polymorphisms.**

The potential interactive effects of polymorphic alleles on serum zinc, plasma IL-6 and MCP-1 and on NK cell lytic activity is shown in Table 4, where NK lytic activity is reported at 1:1 Effector/Target ratio.

A significant interaction of MT1a C- allele with both C- and C+ alleles of IL-6 polymorphism was observed on serum zinc concentrations, that differed significantly before and after supplementation (Analysis of variance  $p=0.05$  at least).

In particular, subjects presenting C- alleles for both polymorphisms (n.7) showed the lowest concentration of basal circulating zinc that was not modified by zinc-aspartate intake. Conversely, MT1a C-/IL-6 C+ carriers (n.7), although their basal concentrations overlapped

those of double C- carriers, were the only ones up-modulated by treatment ( $p=0.003$ , average increase  $2.59\mu\text{mol/L}$ ).

MT1a C+/IL-6 C- ( $n.18$ ) and MT1a C+/IL-6 C+ ( $n.7$ ) carriers showed similar basal concentrations of zinc (over  $11\mu\text{mol/L}$ ) and similar increases after supplementation, very close to the values presented by single C+ polymorphism of MT1a (Figure 1a) and IL-6 (Figure 1b).

Regarding IL-6 plasma concentrations (Table 4), the effect of the two polymorphisms was particularly interesting in the MT1a C-/ IL-6 C+ group, the only one showing a decrease (about 20%) in IL-6 plasmatic concentrations after zinc-aspartate supplementation. All the other polymorphic allele combinations showed similar basal and up-regulated IL-6 concentrations, which in C-C- and C+C+ were significantly increased after supplementation, compared to basal ones.

Concerning MCP-1 (Table 4), all combined polymorphisms showed similar MCP-1 basal concentrations and all, except MT1a C-/ IL-6 C-, tended to down-regulate chemokine concentration after zinc-aspartate supplementation (MT1a C+/IL-6 C+ becoming the relatively lowest, with an approximate 25% decrement compared to basal plasma levels).

Donors with the combined MT 1a C-/IL-6C+ and MT 1a C+/IL-6 C- polymorphisms showed the relatively lowest lytic activity in basal conditions (Table 4), but they underwent a significant increase after zinc supplementation ( $p<0.01$  at least for all the Effector/Target ratios examined, not shown). Conversely, C double negative and C double positive carriers showed only a slight increase in lytic activity (not shown). However, lytic activities after zinc supplementation were generally comparable, with some differences between MT1a C+/IL-6 C- and MT1a C-/IL-6 C- or MT1a C+/IL-6 C+ at the lowest E/T ratio ( $p<0.05$ , not shown).

The interactive effects of polymorphic alleles on circulating zinc concentrations suggested an overloading on pro-inflammatory phenotype due to the concomitant presence of C- alleles of MT1a and IL-6, as seen by the lack of increment in circulating zinc concentrations following zinc-aspartate treatment (Figure 4a), the up-regulation of plasma IL-6 concentrations (Figure 4b), (lower and higher than the single polymorphism ones, respectively, Figure 3b) and the halved increments of NK lytic activity (Figure 4c), compared to single IL-6 C- ones (Figure 3c). Conversely, the pair of MT1a C+ with IL-6 C-, and especially with IL-6 C+, seemed to reduce the pro-inflammatory unbalance (Figure 4b), improving lytic activity (Figure 4c) with the progressive increase of circulating zinc (Figure 4a). Finally, MT1a C-/ IL-6 C+ carriers seemed to be the most favoured by zinc-aspartate treatment; in fact, parallel to the increment

of more than 2  $\mu\text{mol/L}$  circulating zinc (Figure 4a), they showed a reversal between IL-6 plasma concentration (Figure 4b) and NK lytic activity (Figure 4c).

MCP-1 increments (Figure 4d), despite being always negative in single polymorphism carriers, became positive in double negative C carriers and showed an additive decrement in double positive C carriers. In MT1a C+/IL-6 C- and MT1aC-/IL-6 C+, MCP-1 modifications resembled those of C+ allele of the corresponding single polymorphism (Figure 1 e, f).

## DISCUSSION

In this study we evaluated the effect of zinc aspartate supplementation on circulating zinc, IL-6 and MCP-1 plasma concentrations and on NK lytic activity in a healthy elderly population in relationship to some polymorphic allele variants.

The effect of zinc treatment on lytic activity could be due to a conversion from the pro-inflammatory status into a more balanced Th1/Th2 equilibrium, generally Th2 skewed by even mild zinc deficiency (Cakman et al., 1996; Prasad, 2000; Rink et al., 1998). NK lytic activity upmodulation after zinc supplementation was in agreement with previous data demonstrating that zinc deficiency affects NK cells that display decreased number and functional activity as well (Allen et al., 1983; Keen and Gershwin, 1990; Mocchegiani et al., 2003; Prasad, 2000; Ravaglia et al., 2000). Recently, it has been reported that an in vitro zinc stimulation mimicking doses and times of the in vivo supplementation used in this study, fostered a Th1 response probably not activating NK cell directly, but triggering IFN- $\gamma$  synthesis by an increased number of CD56+ NK cells (Metz et al., 2007).

The decrease in plasma concentrations of MCP-1, observed following treatment, agrees with the suggested remodelling to a Th1 prevailing immune response and with the indication that chemokines also participate in the Th2 profile (Mariani et al., 2006). This also indicates that MCP-1 level is influenced by zinc, as demonstrated on lung epithelial cells (Richter et al., 2003) and that zinc supplementation down modulates this chemokine.

Conversely, the effect on IL-6 was unexpected and opposite to that observed for MCP-1, since the treatment with zinc increased plasma concentrations of IL-6 in the overall survey, suggesting an up-regulation of the pro-inflammatory status, even if values remained below the ones observed in chronic diseases. In basal conditions plasma concentrations of IL-6 and MCP-1 were directly correlated, but following zinc supplementation they modulated in opposite ways, approaching a borderline inverse correlation, thus indicating that sensitivity to zinc was probably also dependent on other variables. In any case, the increment of IL-6 was

restricted to a limited biological interval, as suggested by the poor increases observed in subjects with higher basal levels. This effect was not due to gender differences, because circulating zinc and IL-6 concentrations were similar in both sexes.

As concerning a possible reversibility of systemic “low grade inflammation”, as determined by Crp levels, we did not find any influence of zinc supplementation (in agreement with the Monica/Kora study, Scheurig et al., 2007) suggesting that, in our experimental conditions, zinc was able to modulate (positively or negatively) only peculiar pathways and not the overall inflammatory process.

Many recent studies have been given up to the analysis of possible associations among polymorphisms in genes encoding inflammatory mediators, circulating levels of these mediators and longevity. The -174G>C polymorphism has been reported as functionally important since it influences the rate of IL-6 transcription and IL-6 plasma concentration (Brull et al., 2001; Terry et al., 2000). The numerous studies focusing on this polymorphism are very conflicting (see Krabbe et al., 2004, for a mini-review). Some authors suggested that C allele was a risk marker for all-cause mortality, associated with high plasma levels of IL-6 (Krabbe et al., 2004). Other studies, including one by our group on a large survey (Ravaglia et al., 2005), showed no association between this polymorphism and plasma levels of IL-6, which were increased in C- allele carriers only in the presence of concomitant clinical conditions. In agreement, in the present study we did not observe differences in plasma IL-6 concentration in C- or C+ allele carriers before and after zinc supplementation, the only difference being the up-modulation of C- carriers. Likewise, we did not observe different zinc concentrations between C+ and C-, but C+ carriers appeared to be up-modulated, suggesting that zinc responders were also able to limit plasma IL-6 increase.

Furthermore, also MT1a polymorphism seems to influence the observed modifications, in line with the MT role as intracellular distrainers of zinc. In fact, the analysis of MT1a polymorphism showed essential circulating zinc differences between C- and C+ allele carriers, both before and after supplementation, C- allele carriers displaying the lowest levels of zinc without evident increases after supplementation joined to the lowest up-modulation of plasma IL-6. These data are partially in disagreement with a previous study performed on a female survey from central Italy, describing MT1a C+ carriers as characterized by a pro-inflammatory phenotype (high IL-6, low circulating zinc, high intracellular zinc, low zinc bioavailability) compared to C- (Cipriano et al., 2006). Excluding gender and numerosity of the sample, the main difference probably explaining the divergent results was represented by

circulating zinc concentrations that were normal in the previous study (Cipriano et al., 2006) but markedly decreased in the present. Therefore, we suggest that important determinants are also the initial zinc concentration, the zinc/inflammation level and the increment necessary to recover normal basal levels. Some discrepancies might have also resulted from the complex interaction between life style and genetic factors together with cultural, dietetic and genetic differences across countries but also from the study of a single polymorphism rather than the association of more polymorphisms of proteins related to inflammation.

Indeed, the association of the two polymorphisms indicated the C- double carriers as unable to respond to the pharmacological treatment and, concomitantly, unable to limit the over-production of IL-6 and to improve NK activity. On the other side, MT1a C-/IL-6 C+ and carriers were the best responders to zinc supplementation and the only ones able to down modulate IL-6, thus favouring the NK-mediated response, therefore showing a lower degree of inflammatory status, probably for a better zinc release from MT. Thus, the inflammatory response might be intense, long lasting or not modulated in subjects with a given genetic background, as our data suggest for MT1a C-/IL-6 C-, indicating the opportunity to avoid ineffective supplementation in particular patient groups.

In conclusion, in this paper we have shown that zinc-aspartate supplementation in subjects with low or borderline normal circulating zinc levels increases the concentration of this ion and modulates IL-6 and MCP-1 plasma concentrations and NK lytic activity that seem to be differently influenced by the interaction of some polymorphic alleles. Therefore, we suggest that, together with peak IL-6 plasma concentrations reached after an inflammatory response, also the reciprocal changes undergone by the different factors towards the Th1 profile, together with the genetic background, could be important determinants for evaluating the efficacy of zinc treatment and for identifying groups of subjects that can take advantage of therapeutic intervention.

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ACCEPTED MANUSCRIPT



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**TABLE 1**

Selected characteristics of healthy old subjects.

	Total subjects (n=39)	Women (n=19)	Men (n=20)
Age (years)	72.7 ± 6.40	71.9 ± 7.41	73.4 ± 5.46
Zinc (µmol/L)	10.5 ± 1.45 (10.00 – 15.00) *	10.8 ± 1.25	10.2 ± 1.58
Albumin (g/dl)	4.2 ± 0.40 (3.50 – 5.30) *	4.2 ± 0.47	4.2 ± 0.32

Values are expressed as means ± SD. Comparisons between women and men by Mann-Whitney U test did not show significant differences.

\* Normal range values of serum concentrations from a normal young adult population are reported in parentheses and obtained from Trumbo et al., 2001.

**TABLE 2**

Plasma IL-6 and MCP-1 concentrations in basal conditions and after zinc supplementation.

Soluble factors	Conditions	Total subjects (n=39)	Women (n=19)	Men (n=20)
IL-6 (pg/ml)	basal	24.4 ± 2.79 <u>(7.1 ± 5.7)*</u>	23.4 ± 3.80	25.3 ± 4.15
	after zinc	33.3 ± 2.11 <sup>a</sup>	34.1 ± 3.24	32.7 ± 2.78
MCP-1 (pg/ml)	basal	136.4 ± 8.82 <u>(43.16 ± 4.92)*</u>	146.5 ± 13.40	126.8 ± 11.53
	after zinc	116.4 ± 5.63 <sup>b</sup>	116.4 ± 9.02 <sup>b</sup>	116.4 ± 7.21

Values are expressed as means ± SEM.

\* Serum concentrations from 21 normal adult donors (age range 24-55 years) are reported in parentheses (personal unpublished data).

Comparisons between women and men by Mann-Whitney U test did not show significant differences. Comparisons between basal and after zinc supplementation concentrations by Wilcoxon's matched pairs test show significant differences: <sup>a</sup> p<0.05; <sup>b</sup> p<0.01.

**TABLE 3**

NK lytic activity in basal conditions and after zinc supplementation.

E/T cell ratios	Women (n=19)		Men (n=20)	
	NK lytic activity (%)		NK lytic activity (%)	
	basal	after Zn	basal	after Zn
E/T 50	23.0 ± 1.53	29.0 ± 3.40 <sup>b</sup>	22.6 ± 1.91	28.0 ± 3.80
E/T 25	18.8 ± 4.12	25.8 ± 2.56 <sup>b</sup>	18.3 ± 1.79	26.1 ± 2.74 <sup>b</sup>
E/T 12	15.2 ± 1.74	22.1 ± 2.41 <sup>c</sup>	15.1 ± 1.80	22.6 ± 2.47 <sup>c</sup>
E/T 6	12.8 ± 1.68	19.7 ± 2.52 <sup>c</sup>	11.9 ± 1.65	20.2 ± 2.35 <sup>c</sup>
E/T 3	10.9 ± 1.56	18.3 ± 2.14 <sup>c</sup>	10.1 ± 1.47	17.9 ± 1.94 <sup>b</sup>
E/T 1	7.7 ± 1.11	16.0 ± 1.85 <sup>c</sup>	6.1 ± 0.89	15.4 ± 1.86 <sup>a</sup>

Values are expressed as means ± SEM. Comparisons between women and men by Mann-Whitney U test did not show significant differences. Comparisons between basal and after zinc supplementation percentages of NK lytic activity by Wilcoxon's matched pairs test show significant differences: <sup>a</sup>p<0.01; <sup>b</sup>p<0.001; <sup>c</sup>p<0.0005.

**TABLE 4**

Plasma zinc, IL-6 and MCP-1 concentrations according to polymorphic allele combinations : effect of zinc supplementation.

Concentrations	+ 647 MT1a alleles	-174 IL-6 alleles	
		C-	C+
Basal circulating zinc ( $\mu\text{mol/L}$ ) <sup>a</sup>	C-	9.59 $\pm$ 0.60	9.93 $\pm$ 0.37
	C+	11.09 $\pm$ 0.32	11.16 $\pm$ 0.56
Circulating zinc after supplementation ( $\mu\text{mol/L}$ ) <sup>b</sup>	C-	9.50 $\pm$ 0.92	12.53 $\pm$ 0.38
	C+	11.88 $\pm$ 0.41	12.23 $\pm$ 0.46
Basal plasma IL-6 (pg/ml)	C-	18.92 $\pm$ 5.83	28.10 $\pm$ 5.68
	C+	22.61 $\pm$ 5.39	22.73 $\pm$ 7.49
Plasma IL-6 after supplementation <sup>c</sup> (pg/ml)	C-	34.29 $\pm$ 4.14	22.75 $\pm$ 5.33
	C+	32.45 $\pm$ 2.95	39.66 $\pm$ 2.45
Basal plasma MCP-1 (pg/ml)	C-	111.93 $\pm$ 26.59	144.47 $\pm$ 20.59
	C+	122.17 $\pm$ 10.74	123.32 $\pm$ 15.84
Plasma MCP-1 after supplementation (pg/ml)	C-	118.29 $\pm$ 18.92	128.22 $\pm$ 16.25
	C+	108.09 $\pm$ 5.07	97.79 $\pm$ 15.72
Basal NK activity (%)	C-	13.69 $\pm$ 2.98	7.98 $\pm$ 2.19
	C+	6.49 $\pm$ 0.84	11.53 $\pm$ 2.2
NK activity after supplementation (%)	C-	16.25 $\pm$ 2.77	15.80 $\pm$ 2.86
	C+	11.37 $\pm$ 1.27	19.03 $\pm$ 3.85

Values are expressed as means  $\pm$  SEM. Analysis of variance: <sup>a</sup> p=0.05; <sup>b</sup> p=0.006; <sup>c</sup> p=0.06

Basal circulating zinc: MT1a C-/IL-6 C- vs. MT1a C+/IL-6 C- and vs. MT1a C+/IL-6 C+ p<0.05;

Circulating zinc after supplementation: MT1a C-/IL-6 C- vs. MT1a C+/IL-6 C- p<0.003; MT1a C-/IL-6 C- vs. MT1a C+/IL-6 C+ and vs. MT1a C-/IL-6 C+ p<0.02;

Basal circulating zinc vs. zinc after supplementation: MT1a C-/IL-6 C+ p<0.003;

Basal plasma IL-6 vs. plasma IL-6 after supplementation: MT1a C-/IL-6 C- and MT1a C+/IL-6 C+ p<0.05;

Basal NK activity: MT1a C+/IL-6 C- vs. MT1a C-/IL-6 C- and MT1a C+/IL-6 C+ p<0.05;

NK activity after supplementation: MT1a C+/IL-6 C- vs. MT1a C+/IL-6 C+ p=0.07;

Basal NK activity vs. NK activity after supplementation: MT1a C+/IL-6 C- p<0.003 and MT1a C-/IL-6 C+ p<0.01.

**LEGENDS FOR FIGURES**

**Figure 1:** Plasma zinc, IL-6 and MCP-1 concentrations according to +647 MT1a and -174 IL-6 polymorphic alleles: effect of zinc supplementation.

Values are expressed as means  $\pm$  SEM. Comparisons between C- and C+ alleles by Mann-Whitney U test and between basal and after zinc supplementation concentrations by Wilcoxon matched pairs test show significant differences: C- vs. C+ <sup>a</sup>p<0.001; <sup>b</sup>p<0.05; basal vs. after supplementation <sup>c</sup>p<0.001; <sup>d</sup>p<0.005.

**Figure 2:** Lytic activity of NK cells according to +647 MT1a and -174 IL-6 polymorphic alleles: effect of zinc supplementation.

Values are expressed as means  $\pm$  SEM for the different Effector/Target cell ratios; comparisons between basal and after supplementation percentages by Wilcoxon matched pairs test: p<0.05 at least.

**Figure 3:** Increments after supplementation of circulating zinc (a), plasma IL-6 (b) and MCP-1 (d) concentrations, and NK lytic activity (c) in single +647 MT1a and -174 IL-6 polymorphic allele carriers. <sup>a</sup>Zinc concentration: IL-6 C- vs. IL-6C+ p=0.02.

**Figure 4:** Increments after supplementation of circulating zinc (a), plasma IL-6 (b) and MCP-1 (d) concentrations and NK lytic activity (c): interactive effects of combined +647 MT1a and -174 IL-6 polymorphisms. Analysis of variance for zinc concentrations: p<0.02; post hoc: MT1a C-/IL-6 C+ vs. <sup>a</sup>MT1a C-/IL-6 C- p= 0.002 and vs. <sup>b</sup>MT1a C+/IL-6 C- p=0.02.



Figure 1

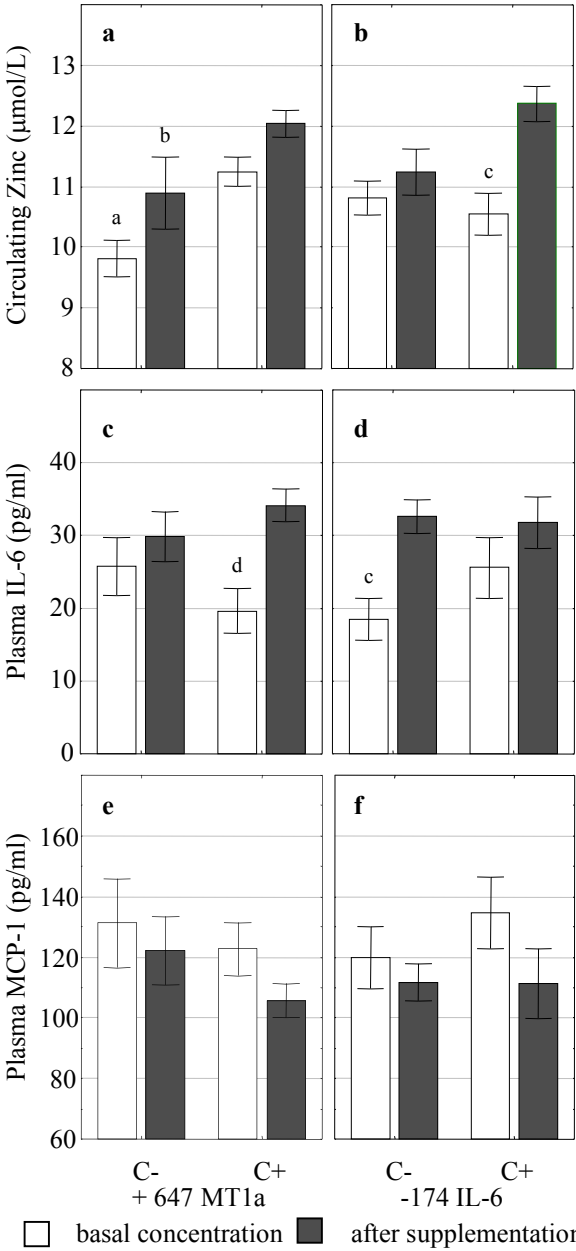


Figure 2

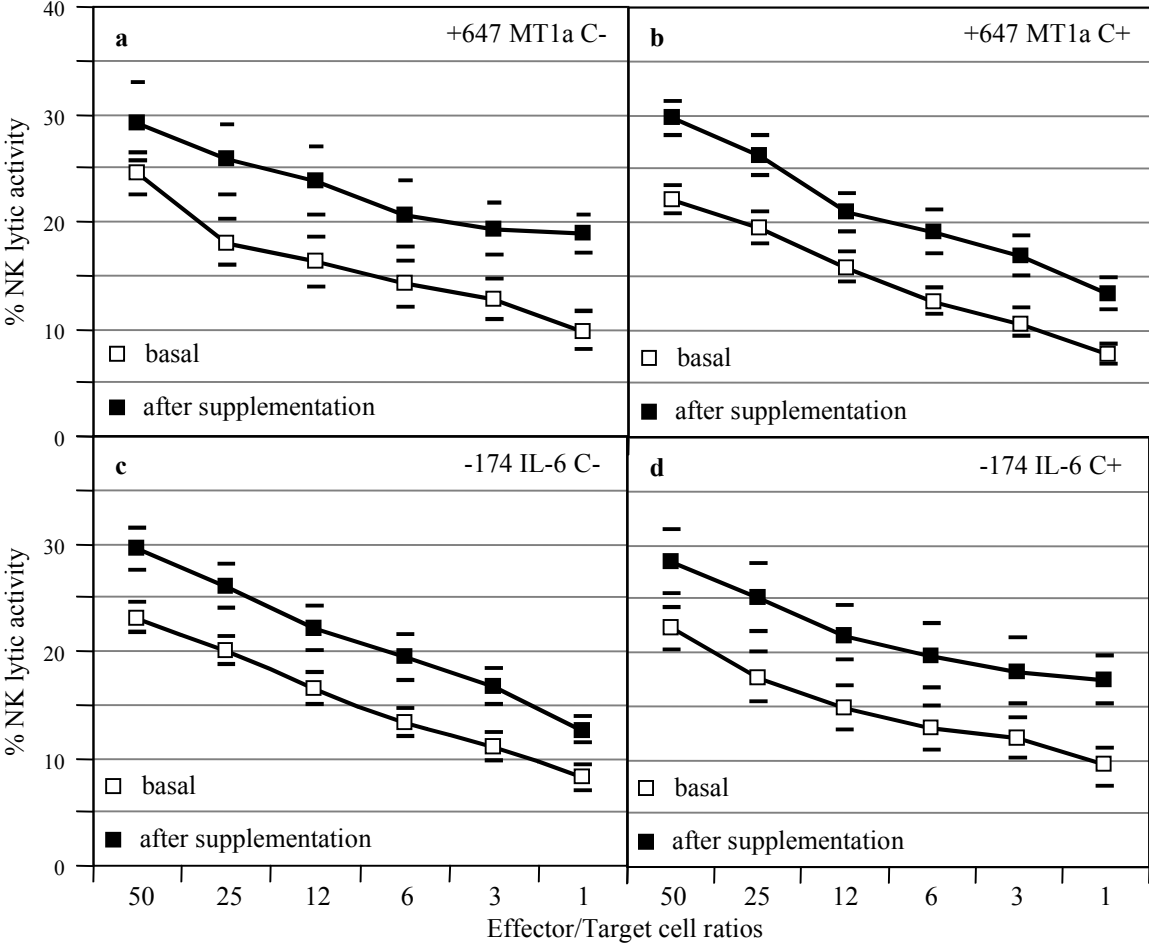


Figure 3

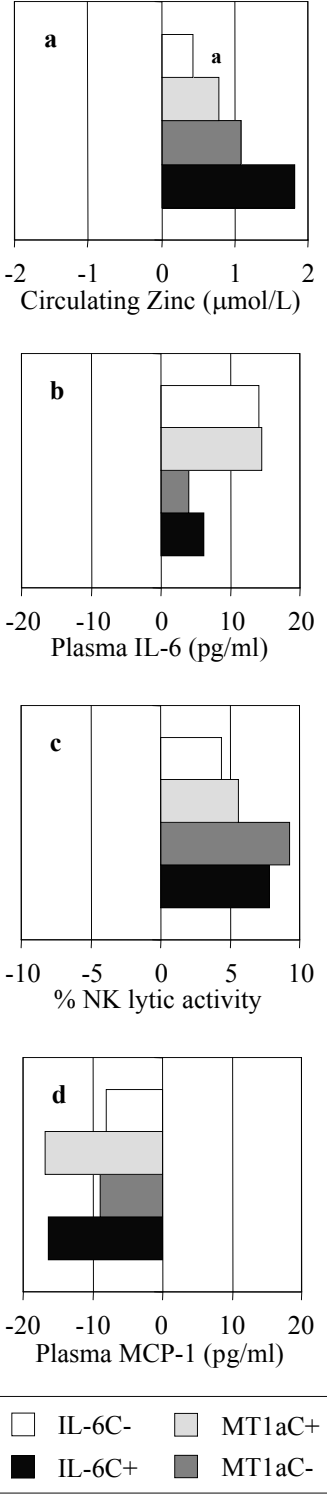


Figure 4

