



## **Multimicronutrient supplementation in older persons decreased zinc deficiency but not serum TNF- $\alpha$**

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### **ABSTRACT**

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The increase in the population of older persons needs to be accompanied by increased quality of healthcare in older persons, particularly a decrease in the incidence of infections. Impaired immune responses are common in older adults, and immune senescence likely contributes to the increased incidence of infectious diseases in the older persons. The aging process decreases the immune response and many studies have been conducted to explain the role of supplementation with various micronutrients, such as vitamin C, vitamin E,  $\beta$ -carotene and zinc, on the immune response. The aim of the present study was to assess the influence of 6 months of multi-micronutrient (MMN) supplementation on zinc and tumor necrosis factor (TNF- $\alpha$ ) levels in older persons. A randomized controlled trial was conducted on 78 older persons, who were divided into two groups. The treatment group received MMN supplementation containing 40 mg elemental zinc, 120 mg ascorbic acid, 6 mg  $\beta$ -carotene, 15 mg  $\alpha$ -tocopherol and 400  $\mu$ g folic acid and the control group 400 mg calcium carbonate. The study did not demonstrate that 6 months of MMN supplementation resulted in significant differences between both groups, with respect to total protein, albumin, globulin, and TNF- $\alpha$  levels. In contrast, MMN supplementation significantly decreased the proportion of older persons with zinc deficiency in the treatment group, in comparison with the control group. The present data suggest that in older persons with relatively good immune and protein status, improvement of the immune status by MMN supplementation may be difficult and at best limited.

**Key words:** Multimicronutrients, TNF- $\alpha$ , protein, zinc, older persons

### **INTRODUCTION**

Technological advances and improved community healthcare services have led to an increased number of patients surviving fatal conditions, resulting in a steadily growing

population of older persons. In the year 2025 the world population of older persons is predicted to exceed one billion, with the majority living in developing countries.<sup>(1)</sup> In Indonesia, the number of older persons in the year 2000 was 14.6 million, increasing to 19.1

million in 2010, and estimated to reach 29.9 million in 2025.<sup>(2)</sup>

The increase in the population of older persons has led to the need for measures to improve their quality of health, particularly by reducing their risk of infection. Older persons generally have an increased risk of infection and a decreased functioning of the immune system. They tend to have increased morbidity of chronic diseases concurrently with the decline in organ functions due to the aging process. One study reported that 50-80% of older persons aged 65 years and over suffer from more than one chronic disease, thus affecting their quality of health.<sup>(3,4)</sup> Nutritional factors also play a role in the health status of older persons and are particularly important for immune functions.<sup>(5)</sup> Micronutrient supplementation has been mostly associated with its effect on immunity and infectious disease. In malnutrition, which is commonly accompanied by micronutrient deficiency, many abnormalities of immune function have been encountered. According to surveys performed in India, Europe, the United States, and Canada, around 35% of older persons have micronutrient deficiencies.<sup>(6)</sup>

For an efficiently functioning immune system, adequate micronutrient intakes are required, as micronutrient deficiencies depress immunity. This condition causes the body to become more susceptible to infection, thus increasing morbidity and mortality in older persons. Conversely, infection may also cause micronutrient deficiencies due to reduced nutrient intakes or through changes in metabolic pathways.<sup>(7)</sup>

Oxidative stress has long been recognized as an important factor in several infectious diseases associated with degenerative processes, such as atherosclerosis, cardiac disorders, neurodegenerative disease, immunological disorders, and even the aging process itself.<sup>(8)</sup> Various reactive oxygen species (ROS) have been recognized, i.e.  $O_2^{\cdot-}$ ,  $H_2O_2$ , and  $\cdot OH$ , which are continuously generated *in vivo* under

aerobic conditions. Inflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) have long been known to generate ROS in large numbers.<sup>(8)</sup> TNF- $\alpha$  is a proinflammatory cytokine mainly produced by activated macrophages. Several studies have demonstrated that zinc deficiency affects the production or the biological activity of TNF- $\alpha$ . On the other hand, zinc supplementation given to normal healthy subjects and to cardiac patients resulted in reduced oxidative stress and serum TNF- $\alpha$  levels.<sup>(9,10)</sup>

However, the effect of micronutrient supplementation on serum zinc, protein and TNF- $\alpha$  levels in healthy older persons has not been fully clarified. Aging is associated with decreased immune responses and increased frequency of infections, and it may be assumed that older persons suffer from inapparent infections, such that they can be said to have received "natural vaccination". Infection causes T-cell activation, accompanied by the release of serum TNF- $\alpha$ , such that serum TNF- $\alpha$  levels can be taken as a parameter of infection. Advancing age is probably also associated with failure of control of TNF- $\alpha$  production, and plays a role in infectious events.<sup>(11)</sup> Any beneficial effect of multi-micronutrient (MMN) supplementation would be expected to increase serum zinc and protein levels, while decreasing TNF- $\alpha$  levels. Therefore the aim of the present study was to determine whether MMN supplementation in naturally immunized older persons increased serum zinc and protein levels, and decreased TNF- $\alpha$  levels.

## METHODS

### Research design

This study was an experimental study designed as a randomized double blind controlled trial, conducted in Mampang Prapatan subdistrict, South Jakarta, from 1 July 2006 up to 28 February 2007.

## Subjects

The target population comprised older persons resident in Mampang Prapatan subdistrict, South Jakarta. Inclusion criteria were: male and female older persons aged 60 years and older, in good health, capable of communication, capable of walking without support, and willing to participate in the present study. Exclusion criteria were individuals with severe or terminal disease, and patients with diabetes mellitus. The sample size was expected to detect a 15% difference in plasma TNF- $\alpha$  concentration, with a 2-sided *p* value of 0.05, and an approximate power of 80%. The sample size was computed statistically and was determined to comprise 80 subjects. Sample selection was by multistage cluster and simple random sampling.

## Randomization

The subjects were randomly assigned into the MMN supplemented or the control group, using envelopes, each of which contained 2 smaller envelopes (1 assigning a subject to MMN treatment and 1 assigning a subject to placebo). The MMN group received a supplement containing 40 mg elemental zinc (as zinc gluconate), 120 mg ascorbic acid, 6 mg  $\beta$ -carotene, 15 mg  $\alpha$ -tocopherol (d- $\alpha$ -tocopheryl acid succinate) and 400  $\mu$ g folic acid. The control group received a preparation containing 400 mg calcium carbonate. Random allocation was performed by means of a block-of-6 design, for which the sequence of random numbers was generated using Microsoft Excel.

## Intervention

Supplementation was performed once daily up to 6 months and administered by field workers at home visits. Participants in the treatment group received supplements containing MMN, as explained above, and the control group received supplements containing calcium. Both types of supplement were identical in form, color, and taste/ flavor, so as

to be superficially indistinguishable. Subjects were considered to have dropped out of the study after failing to take 10% of supplements or by missing two weeks of supplementation. Both MMN and control capsules were supplied by PT Ikapharmindo Laboratories (Jakarta).

## Data collection

The characteristics of age, gender, educational status, marital status, and health status were collected by interviews using questionnaires that had been tested in a preliminary trial. Collection of subject characteristics was done by field workers at baseline (start of study) and after supplementation for 6 months.

## Measurements

Body mass index (BMI) was calculated by dividing body weight (kg) by height (m) squared. Body weight was determined using Sage scales accurate to 0.1 kg, while height was measured by means of microtoise accurate to 0.1 cm. For Asian populations, BMI is classified into the following categories: underweight (<18.5 kg/m<sup>2</sup>), normal (18.5–22.9 kg/m<sup>2</sup>), overweight (23.0–27.5 kg/m<sup>2</sup>), and obese (>27.6 kg/m<sup>2</sup>).<sup>(12)</sup>

## Biochemical measurements

From each subject 10 mL of venous blood was collected after an overnight fast of 10 hours, for laboratory determination of total protein, albumin and globulin, hemoglobin, zinc and TNF- $\alpha$ . Determinations were performed twice, i.e. before and after 6 months of supplementation. Measurement of TNF- $\alpha$  concentration (pg/mL) was performed by means of an ELISA kit (Invitrogen). Assessment of total protein (g/dL), albumin (g/dL) and globulin (g/dL) was also done using ELISA. Plasma Zn levels were determined by flame atomic absorption spectrometry using standard methodology. A cut-off of 10.7  $\mu$ mol/L zinc was taken as an index of zinc deficiency.<sup>(13)</sup>

### Ethical clearance

Older persons willing to participate in the study were informed of the aims and protocol of the study. Each of the subjects was informed that their participation in this study was voluntary and that they had the right to withdraw at will from the study at any time without giving any reasons. After the subjects understood the information given and were willing to participate, they were asked to sign an informed consent form approved by the Commission on Research Ethics of the Medical Faculty, Trisakti University.

### Statistical analysis

Demographic differences between MMN and control groups were examined by t-test (age) and chi-square test (gender, educational level, and marital status). For other variables, t-tests were used to compare group differences when variables were normally distributed. Analyses were performed with SPSS for Windows, version 15.0 (SPSS Inc, Chicago,

IL). The manufacturer of the supplements was not involved in the study design and data analysis. The authors had full and unrestricted rights to analyze, interpret, and publish the results.

### RESULTS

From 1 July 2006 to 28 February 2008, a total of 100 older persons underwent screening. As shown in Figure 1, 90 older persons as study subjects were randomly assigned to two treatment groups: 45 were assigned to the MMN group and 45 were assigned to the control group. After 6 months of intervention, 12 older persons dropped out of the study (3 in the MMN group and 9 in the control group). The most common reason for withdrawal was gastrointestinal upset (2 subjects in the MMN group and 6 subjects in the control group). A total of 42 subjects in the MMN group and 36 subjects in the control group participated in the study up to completion and all were included in the data analysis.

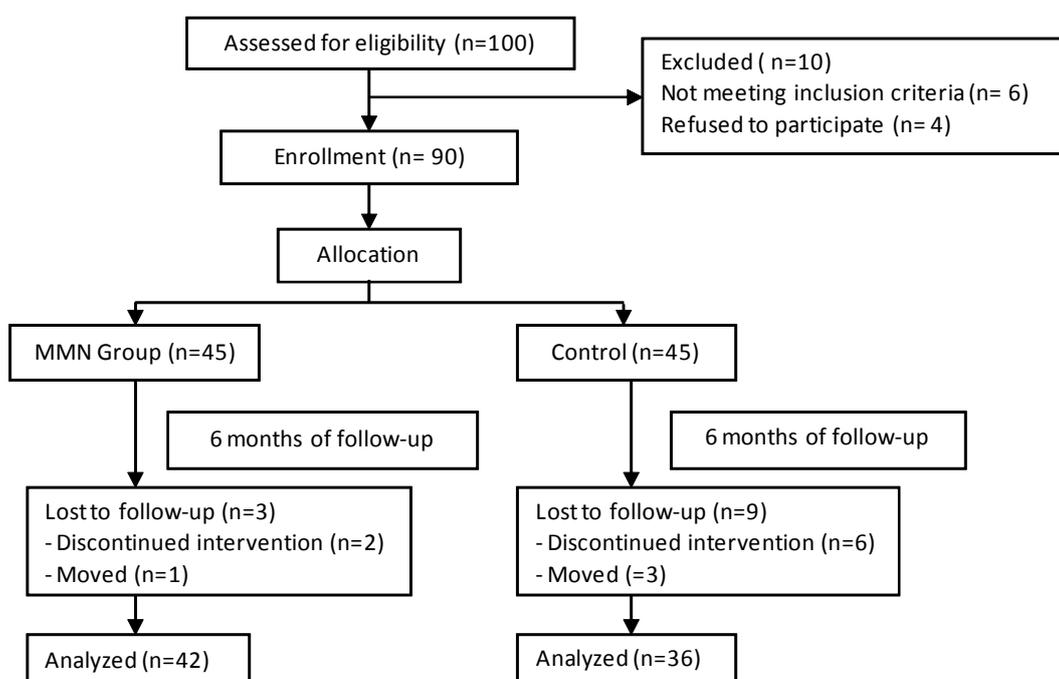


Figure 1. Flowchart of the study design and subject participation throughout the study (MMN = multi-micronutrient)

Table 1. Baseline descriptive statistics of the subjects by treatment groups

Variable	Supplement (n=42)	Control (n=36)	P
Gender (%)			
Male	38.1	33.3	0.813 <sup>†</sup>
Female	61.9	66.7	
Age (yrs) <sup>§</sup>	65.5 ± 4.4	65.0 ± 4.5	0.601 <sup>†</sup>
Education (%)			
No education	28.6	22.2	
Primary education	50.0	58.3	0.554 <sup>†</sup>
Junior high school	14.3	8.3	
Senior high school	4.8	11.1	
Tertiary education	2.4	0	
Marital status (%)			
Married	35.7	47.2	0.359 <sup>†</sup>
Widowed	64.3	52.8	
Body mass index <sup>§</sup>	21.8 ± 4.5	22.1 ± 4.1	0.874
Underweight	26.2	25.0	
Normal	28.6	38.9	0.359
Overweight	35.7	19.4	
Obese	9.5	16.7	

<sup>†</sup>p values by chi-square test; <sup>†</sup>p value by independent t-test; <sup>§</sup>Mean ± SD

Baseline characteristics of the study subjects are listed in Table 1. Age, gender, level of education, marriage status were similar at baseline among the treatment groups ( $p > 0.05$ ). Baseline mean BMI was not significantly different in both study groups ( $p = 0.874$ ). Compliance with the study protocol was confirmed by pill counts and about 95% of the subjects in the two groups had good compliance.

Serum total protein, albumin, globulin and TNF- $\alpha$  levels at baseline are presented in Table 2. Obtained values of total protein, albumin, globulin and TNF- $\alpha$  levels were not significantly

different in both groups ( $p > 0.05$ ). The proportion of subjects with zinc deficiency was significantly higher in the MMN group (50%) in comparison with the control group (13.9%) ( $p = 0.01$ ). Baseline zinc levels in the MMN group ( $11.24 \pm 2.01 \mu\text{mol/L}$ ) were lower than those in the control group ( $12.19 \pm 1.59$ ) ( $p = 0.024$ ). The number of MMN subjects with zinc deficiency was significantly higher than the control subjects ( $p = 0.01$ ).

After administration of 6 months of MMN supplementation, values for total protein, albumin, globulin, and TNF- $\alpha$  levels were not

Table 2. Zinc, protein and TNF- $\alpha$  levels at baseline, by treatment groups

Variable	Supplement (n=46)	Control (n=36)	P
Total protein (g/dL) <sup>§</sup>	7.15 ± 1.07	7.11 ± 1.17	0.367 <sup>†</sup>
Albumin (g/dL) <sup>§</sup>	3.97 ± 0.61	3.89 ± 0.60	0.952 <sup>†</sup>
Globulin (g/dL) <sup>§</sup>	3.18 ± 0.63	3.24 ± 0.67	0.846 <sup>†</sup>
TNF- $\alpha$ (pg/mL) <sup>§</sup>	2.32 ± 2.11	1.89 ± 2.25	0.670
Zinc ( $\mu\text{mol/L}$ ) <sup>§</sup>	11.24 ± 2.0	12.19 ± 1.59	0.024 <sup>*†</sup>
Zinc deficiency (<10.7 $\mu\text{mol/L}$ )	50.0%	13.9%	0.011 <sup>*†</sup>

<sup>†</sup>p values by independent t-test; <sup>†</sup>p value by chi-square test; \* Significance; <sup>§</sup> Mean ± SD

Table 3. Zinc, protein and TNF- $\alpha$  levels after 6 months of supplementation, by treatment groups

Variable	Supplement (n=46)	Control (n=36)	P
Total protein (g/dL) <sup>§</sup>	7.82 $\pm$ 0.44	7.84 $\pm$ 1.41	0.415 <sup>†</sup>
Albumin (g/dL) <sup>§</sup>	4.71 $\pm$ 0.35	4.68 $\pm$ 0.25	0.513 <sup>†</sup>
Globulin (g/dL) <sup>§</sup>	3.11 $\pm$ 0.49	3.16 $\pm$ 0.45	0.157 <sup>†</sup>
TNF- $\alpha$ (pg/mL) <sup>§</sup>	2.38 $\pm$ 2.01	1.89 $\pm$ 2.05	0.514 <sup>†</sup>
Zinc deficiency (<10.7 $\mu$ mol/L)	33.3%	13.9%	0.046 <sup>*†</sup>

<sup>†</sup>p value by independent t-test; <sup>‡</sup>p value by chi-square test; \* Significance; <sup>§</sup>Mean  $\pm$  SD

significantly different between MMN and control groups ( $p > 0.05$ ) (Table 3). In the group receiving MMN supplementation for 6 months the proportion of subjects with zinc deficiency was reduced to 33.3%, while that in the control group remained at baseline level.

## DISCUSSION

Our study showed that MMN supplementation administered to older persons for six months resulted in a nonsignificant effect on plasma total protein, albumin, globulin, and TNF- $\alpha$ . Differing results were found in the study by Prasad et al. on oral supplementation of zinc gluconate (45 mg elemental Zn/d) for 12 months to 49 subjects in the age range of 55-87 years. Their study found that the ex vivo generation of TNF- $\alpha$  decreased significantly in the zinc supplemented subjects and increased significantly in the placebo group. It was also observed that TNF- $\alpha$  in the placebo group increased with time, suggesting that this increase may be an effect of increasing age.<sup>(8)</sup> These differences in outcomes presumably occurred because our study subjects were not vaccinated against influenza and pneumonia, unlike the subjects in Prasad's study,<sup>(8)</sup> in whom vaccination against influenza and pneumonia stimulated their immune system. A few years previously, Prasad et al.<sup>(9)</sup> were the first to demonstrate that zinc supplementation to healthy volunteers significantly decreased oxidative stress. In their study, oxidative stress

was assessed by lipid peroxidation and DNA oxidation, and also by determining NF- $\kappa$ B activation induced by TNF- $\alpha$  in cultures of mononuclear cells isolated from the subjects. A more recent study in Turkey evaluated the effect of zinc supplementation in children with heart disease at a dose of 30 mg elemental zinc given for 28 days after administration of influenza vaccine. Among 49 recruited subjects, a total of 44 children completed the study. The results of this Turkish study showed that zinc supplementation significantly decreased serum TNF- $\alpha$  levels, compared with the non-supplemented group.<sup>(10)</sup>

On the other hand, our finding that MMN supplementation did not affect plasma protein and TNF- $\alpha$  levels was similar to the results of a study by Albers et al.<sup>(14)</sup> using a murine sensitization model. The investigators studied the effects of micronutrient supplementation on markers of adaptive and innate responses, including serum TNF- $\alpha$ , after sensitization and subsequent challenge of the experimental animals with di-nitro-chlorobenzene (DNCB). The diets of the experimental animals were supplemented with a single antioxidant, comprising either one of the vitamins A, C, and E, or either one of the minerals zinc and selenium. There were no significant differences in TNF- $\alpha$  levels between all antioxidant groups and the control group. The TNF- $\alpha$  level for the zinc group was 164  $\pm$  15 pg/mL, compared to 175  $\pm$  13 pg/mL for the control group.

The inability of MMN supplementation to improve the immune functions of our study subjects may have been due to the large number of zinc-deficient subjects. At baseline, 50% of the subjects in the MMN group had zinc deficiency, but after 6 months of MMN supplementation the proportion of zinc deficient subjects, although reduced, still amounted to 33.3%, in comparison with the control group, where it remained at its baseline level of 13.9%. Many animal studies have shown that zinc deficiency decreases resistance to a wide range of pathogenic microorganisms, probably due to impairment of immune responses.<sup>(15)</sup> The zinc deficiency in our subjects was presumably due to changes in dietary preferences, income, and total energy intake, leading to decreased zinc intake, below the recommended dietary allowance (RDA) of 12–15 mg/d in many older adults.<sup>(16)</sup>

Nutrition is an important determinant of the health of older persons, which is closely associated with increased morbidity of chronic and acute diseases in these persons. Malnutrition in older persons frequently remains undetected; for detection of malnutrition, several of the following parameters need to be evaluated: loss of weight, abnormal body mass index, micronutrient deficiencies, and reduced nutrient intakes.<sup>(17)</sup> The types of undernutrition frequently found in older persons are protein and energy deficiencies. Epidemiological data indicate that 5-10% of older persons have malnutrition, and the prevalence of malnutrition increases up to 26% in hospitalized patients with acute infections.<sup>(18)</sup> The changes in bioavailability also play a role in micronutrient deficiencies of older persons. Around 24-40% of older persons suffer from atrophic gastritis, leading to impaired absorption of micronutrients in older persons.<sup>(19)</sup> Micronutrients are required as cofactors and for modulating enzyme activity in metabolism.<sup>(20)</sup> Micronutrient deficiency may affect metabolic pathways mediated by

various enzymes and for enzyme synthesis adequate levels of protein are required.

In our study, 6 months of MMN supplementation had no effect on total protein, albumin, and globulin levels in the blood. This is likely due to the fact that baseline data did not reveal deficiency of total protein. The aging process results in a reduced immune response in older persons. However, there are other factors apart from the aging process itself that may influence the immune response in older persons. Such factors as environmental factors, exposure to antigens, metabolic processes, and infections, may induce activation of immune cells and release of proinflammatory cytokines.<sup>(15)</sup> The immune response is also affected by micronutrient deficiencies, which are frequently encountered in older persons. Data on a population of home-dwelling older persons of age 75+ years indicate that around one-third to one-half of older persons have micronutrient deficiencies. The study conducted by Fabian et al.<sup>(21)</sup> reported low mean energy and micronutrient intakes in older persons, as compared with young adults. There was a low proportion of protein intake compared with that of carbohydrates, while fat intake tended to be high. In addition, mean intakes of micronutrients, such as calcium, folic acid, vitamins A, B, C and D, and the minerals magnesium, iron, zinc, copper, and manganese, were also low.<sup>(22)</sup> It would be interesting to determine the potential of MMN supplementation for improving immune functions and reducing the incidence of infections in older persons. In the US, supplements are used by about 40% of individuals, including older persons. However, although the prevalence of supplement consumption in older persons in the US is apparently substantial, micronutrient deficiencies are still frequently found in older persons. The factors influencing nutritional disorders in older persons comprise economic, social, mental, environmental, physiological, and health-related factors.<sup>(23)</sup>

The present study did not succeed in demonstrating the expected reduction of TNF- $\alpha$  levels as an outcome of MMN supplementation. These results may be explained by the fact that TNF- $\alpha$  levels are influenced by many other factors in addition to micronutrients. Other influencing factors of equal importance are socio-economic factors and plasma zinc levels. This study was conducted on older persons with poor socio-economic status, zinc deficiency, and low educational status, potentially decreasing the expected outcomes. Furthermore, older persons with or without micronutrient deficiencies have reduced immune responses as a result of the aging process itself.<sup>(25,26)</sup>

One of the limitations of our study was that it did not determine dietary intakes of the subjects, thus was unable to determine their nutritional status. MMN supplementation *an sich* was found to be inadequate for increasing immune function, and plasma protein and zinc levels. Another limitation of this study was the duration of supplementation of less than 12 months, unlike that used by Prasad et al.<sup>(8)</sup>

## CONCLUSIONS

MMN supplementation of zinc, ascorbic acid,  $\beta$ -carotene,  $\alpha$ -tocopherol and folic acid for 6 months did not improve protein and TNF- $\alpha$  levels, but was capable of reducing the proportion of older persons with baseline zinc deficiency. Improvement of immune parameters in individuals with a generally good immune and protein status but poor in zinc levels may be difficult to obtain with nutritional intervention.

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