Original Communication

Influence of a Complex Micronutrient Supplement on the Immune Status of Elderly Individuals

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Abstract: Nutritional status is known to have profound effects on immune function and resistance to infections, particularly in the elderly. We investigated the effect of a complex micronutrient supplement in elderly people on the changes in some of the cellular components of the immune system, on lymphocyte function, and on the antibody response to influenza vaccination.

One-hundred-six subjects aged 62 to 98 were randomly assigned to receive a complex micronutrient supplement or a placebo for three months. Subjects were vaccinated against influenza after eight weeks. Clinical parameters, lymphocyte subsets, *in vitro* lymphocyte activation, and influenza antibody titers were assessed at baseline and after 90 days of supplementation.

A significant increase in total lymphocytes ($p=0.034$) and white blood cells (WBC) ($p=0.03$) in the supplemented group was observed. A shift from CD4+/CD45RO+ "memory" cells to CD4+/CD45RA+ "naïve" T-cells in favor of CD4+/CD45RA+ "naïve" T-cells took place. The group consuming additional micronutrients showed an increase in CD45RA+ subsets ($p=0.032$) compared to the control group. A decrease of total cholesterol (from 228.72 ± 56.11 to 210.74 ± 52.58, $p=0.002$) and low-density lipoprotein (LDL) (from 145.75 ± 48.86 to 125.47 ± 41.72, $p<0.001$) was observed. Influenza antibody titers showed no correlation with micronutrient intake.

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We conclude that supplementation with a complex micronutrient formulation increases the number of various types of immune cells and decreases total cholesterol and LDL in elderly people. No beneficial effect on specific antibody response to influenza vaccination was observed. Further research is needed to evaluate whether enhanced cellular immune responses decrease the incidence of infections in elderly people.

Key words: immunity, elderly individuals, naïve T-cells, immunosenescence, micronutrients

Introduction

Aging is associated with a progressive decline in immune competence and with a greater susceptibility to nutritional deficiencies. Age-related changes in the immune response affect both humoral and cellular components of the immune system [1]. Elderly individuals not only demonstrate greater morbidity and mortality from influenza than young adults, but also have greater difficulty mounting a protective response to influenza vaccine [2], showing delayed peaks and shorter antibody titer maintenance [3]. Age-related immune deficiencies are mainly associated with a decline in T-cell-mediated immunity [4]. This is a multifactorial phenomenon affecting T-cell number, T-cell subset composition, and immune functions including lymphocyte proliferation and cytokine production [5]. Compared with young adults, the immune systems of the elderly demonstrate age-related alterations, including a decreased CD4/CD8 ratio [6] and reduced natural killer (NK) cell cytotoxicity [7,8].

The most striking age-related changes include increased HLA-DR+ T lymphocytes and a shift in expression of CD45 isoforms from the CD45RA+CD45RO- to the CD45RA-CD45RO+ subset [9]. When the peripheral blood T-lymphocytes of centenarians are compared with younger adults, cell surface expression of CD28 is significantly decreased in the elderly [10]. CD28 acts as a co-stimulatory molecule required for optimal activation and proliferation following T-cell receptor activation.

Human T-cell subsets CD4+/CD45RA+ and CD4+/CD45RO+, isoforms of the common leukocyte antigen family, are considered to be markers for the aging immune system [11]. Investigations of CD45 isoform expression on CD4+ and CD8+ cells throughout life show a decrease in CD45RA+ cell numbers and a rapid increase in CD45RO+ cells in the first two to three decades of life following antigen exposure [12]. Gene expression and production of interleukin-2 (IL-2) by T-cells, a cytokine required for proliferation of activated T-lymphocytes, as well as IL-2 receptors, also decline with age [5,13].

The primary immunological change in the elderly reflects the age-dependent intrinsic decline of immune responsiveness that is also observed in healthy persons [9,14]. Secondary immunological changes are due to underlying diseases and environmental factors, including diet, drug exposure, physical activity, or exposure to antigens [14].

Nutritional status is a critical determinant of immune responsiveness, and malnutrition is the most common cause of immunodeficiency worldwide. Malnutrition is associated with a significant impairment of cell-mediated immunity [15]. Micronutrients, such as trace elements, vitamins, antioxidants, and fatty acids are known to mediate immune response in the elderly [16,17].

The immune response in the aged is more sensitive to nutritional status than in younger adults and therefore aging and nutritional deficiency exert a cumulative influence on immune responsiveness [18]. Improved immunocompetence is associated with a decreased rate of infections and a positive antibody response to vaccines [19].

The nutritional supplement used in this trial, to investigate its effects on the immune status of elderly volunteers, is a complex formulation containing wheat sprouts enriched with an electrolyte solution during four days of germination [20], containing highly unsaturated fatty acids, antioxidants, and other plant compounds. A similar formulation with different dosages of micronutrients has been used in an open study in HIV-positive patients over a period of 10 or 15 months. The results of this investigation showed good stabilization of cellular immune-related parameters [21].

Subjects and methods

This was a randomized, placebo-controlled, double-blind, multi-center, prospective trial conducted over a period of three months with a follow-up period of

two months. The study population consisted of 106 residents (17 men, 89 women) from five independent centers of a nursing home. Individuals had been living in the nursing home for at least three months, had a life expectancy of at least six months, and were able to eat and drink without complications. Subjects showed no evidence of autoimmune disease, hematologic neoplasia, or renal insufficiency. No immune-modulating or immune-suppressing therapy was administered before or during the study.

All individuals provided informed consent and the study was approved by the regional ethics committee. The average age of the subjects was 85 (62–98) in the supplemented group and 85.5 (65–98) in the control group. Subjects were vaccinated against influenza using Vaxigrip® (Aventis), containing the vaccine strains A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), and B/Yamanashi/166/98, eight weeks after starting nutritional intervention. Subjects were randomly assigned to receive either the nutrient supplement (n=54) or an isocaloric placebo (n=52). Supplement and placebo were macroscopically identical and were prepared as a powder in four different flavors (tomato, garlic-asparagus, mushroom, and vegetable). They were mixed with hot water before use and served as a soup. Table I shows the composition of the supplement. No additional nutritional therapy was given during the study. The subjects' medication was continued unchanged if applicable. Compliance was monitored by the staff of the nursing home by measuring the actual volume of the consumed supplement or placebo. Compliance was judged to be achieved when subjects consumed more than 50 % of the daily recommended amount within 80 % of the study-period. Body weight, body-mass index (BMI), temperature, blood pressure, electrocardiogram (ECG), Mini-Mental Status, Barthel Index, and the Norton Scale were assessed for each volunteer. All analyses were conducted at the start of the study and after three months of supplement or placebo.

A fasting peripheral blood sample was collected and a complete blood profile and count was obtained from each subject. Blood samples were analyzed by a reference laboratory in Vienna, Austria. Messenger RNA was extracted, amplified, and quantified by real-time-polymerase chain reaction (RT-PCR). Lymphocyte activity was determined by comparing mRNA expression of IL2 with mRNA expression of GADPH with and without lectin stimulation. Interleukin-2 receptors (IL2r) were measured after stimulation with phytohemagglutinin, analyzing 3000 cells by fluorescence-activated cell sorting (FACS™).

Total lymphocytes were collected using TruCount (Becton-Dickinson) and assessed by measuring the amount of CD3+. CD2+/CD3+ and CD2+/CD3- were measured for further lymphocyte identification. Differentiation of lymphocyte subsets: CD3+/CD4+ for T-helper cells, CD3+/CD8+ for T-suppressor cells, CD3-/CD16, 56+ for NK cells, CD3+/HLA-DR+ for activated T-lymphocytes, and CD8+/CD38+ for cytotoxic T-lymphocytes was performed by flow cytometry. To evaluate the aging immune system, CD4+/CD45RA+ and CD4+/CD45RO+ were measured. The quantity of B-lymphocytes was evaluated by measuring CD19+. Antibody response to influenza strains A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), and B/Yamanashi/166/98 was measured by an hemagglutination-inhibition test.

Differences between groups were tested using Student's *t*-test for dependent samples. Student's *t*-test for independent samples was applied for the comparison of baseline values and the comparison of the changes after three months between the two groups. The significance level was set at $p<0.05$. All analyses were conducted with SPSS 9.0 for Windows version 2000 (SPSS GmbH, München).

Results

A total of 82 (67 women and 15 men; nutrient supplement group n=40, placebo group n=42) out of 106 subjects completed the study. Nineteen subjects dropped out for various reasons. Five subjects showed low compliance and their data was not included for evaluation. Body weight, BMI, and blood cytology values before and after the study are shown in Table II. The increase in total leukocytes as well as lymphocytes after three months was statistically significant between the two groups (leukocytes p=0.03, lymphocytes p=0.034) and was attributed to the test preparation.

Table III depicts changes in lymphocyte subsets. CD3+ and CD2+/CD3+ cells were significantly influenced by micronutrient supplementation (p=0.023 and p=0.037, respectively) compared to controls. A significant difference after three months could be observed in the supplemented group with respect to CD4+ cells compared to baseline (p=0.014). CD8+ cells increased significantly after three months in the supplemented group (p=0.003). But no difference was observed in the number of CD8+ cells when comparing the groups (p=0.061).

Table I: Supplement micronutrient composition

Component	Dose		Component	Dose		Component	Dose		Component	Dose	
PANMOL®-Sprouts	5000	mg	Potassium	266.5	mg	**Omega-3 fatty acids**			p-Aminobenzoic acid	4.2	mg
Vitamins			Cobalt	49	mcg	Alpha-Linolenic acid	889.7	mg	**Others**		
Biotin	300	mcg	Copper	0.97	mg	**Omega-6 fatty acids**			Betaine	4.2	mg
B 1	6.6	mg	Lithium	1.1	mg	Linolenic acid	1428.6	mg	Carbonate	637	mg
B 2	9.0	mg	Magnesium	157.4	mg	Gamma-Linolenic acid	209.3	mg	Hexacosanol	41.6	mcg
B 6	9.6	mg	Manganese	2,1	mg	**Omega-7 fatty acids**	6.0	mg	Hydrogen carbonate	213	mg
B 12	6	mcg	Molybdenum	48	mcg	**Omega-9 fatty acids**	704.9	mg	Inosine	4.2	mg
C	378.1	mg	Sodium	1731.1	mg	**Other fatty acids**	787.5	mg	Inositol	12.5	mg
E	237.0	mg	Phosphorus	112.8	mg	**Plant Compounds**			L-Glutamine	8.3	mg
Folic acid	196	mcg	Selenium	57	mcg	Anthocyanins	27.5	mg	N-Acetylcysteine	41.7	mg
K1	25	mcg	Silicon	2144.2	mg	Bioflavonoids	27.5	mg	Octacosanol	166.5	mcg
Niacin	60	mg	Vanadium	63	mcg	Carotenoids	162.9	mcg	red. Glutathione	15	mg
Pantothenic acid	30	mg	Zinc	8.3	mg	Isoflavones	75	mg	Taurine	25	mg
Minerals and trace elements			**Phospholipids**			Lignans	43	mcg	Tetracosanol	83.3	mcg
Calcium	319.3	mg	Total lecithin	500	mg	Phytosterins	3.7	mg	Triacosanol	208.1	mcg
Chloride	2550	mg	Phosphatidylcholine	110	mg	Quercetin	27.5	mg			
Chromium	53	mcg	Phosphatidylethanolamine	100	mg	**Vitaminoids**					
Iron	7.7	mg	Phosphatidylinositol	67.5	mg	Alpha-Lipoic acid	15.0	mg			
Fluorine	0.15	mg	Phosphatidic acid	30	mg	Coenzyme Q 10	4.5	mg			
Iodide	30	mcg	Choline	17.3	mg	Orotate	4.2	mg			

Table II: Characteristics of treatment groups at baseline and after 3 month (Mean ± SD)

	Supplement		Control	
	Baseline	3 months	Baseline	3 months
Body weight (kg)	67.0±10.62	68.5±10.99	59.5±14.58	57.5±15.17
BMI (kg/m^2)	25.96±4.117	26.3±4.268	24.4±4.861	24.1±5.219
White blood cell count	6044.50±1769.3	7178.72±4172.91*	6322.86±1685.36	6015.00±1893.40
Lymphocyte count	2003.68±800.11	2270.51±924.78†	2005.95±641.05	1983.10±665.32
Body temperature	36.278±0.336	36.173±0.319	36.245±0.334	36.19±0.438
CRP	1.003±1.278	1.829±4.467	1.036±1.218	1.106±0.877
Concomitant medication	5.35±2.61	6.08±3.02	4.05±2.16	4.71±2.63

Note: BMI=body mass index; weight/height^2; CRP: C-reactive protein
n=40 in the supplement group and n=42 in the control group
* significantly different from baseline (p=0.03)
† significantly different from control group (p=0.034)

Table III: Lymphocyte subsets in subjects before and after micronutrient supplementation compared to control group (Mean±SD)

	Supplement		Control		
	Baseline	3 months	Baseline	3 months	P value[a]
CD2+/CD3+	1418.80±587.81	1650.10±731.04*,†	1469.98±540.54	1463.52±523.10	0.037
CD2+/CD3-	253.08±160.04	279.05±201.55	246.14±103.83	264.02±140.76	0.692
CD3+	1403.75±595.45	1628.00±738.05*,†	1466.38±535.50	1471.60±531.87	0.023
CD3+/CD4+	995.45±363.40	1095.59±486.57*	945.38±368.86	963.33±363.31	0.093
CD3+/CD8+	451.43±306.75	561.87±354.21*	485.79±422.49	497.81±351.99	0.061
CD3-/CD16,56+	335.35±218.09	385.59±250.69*	309.14±131.18	342.10±168.77	0.509
CD3+/CD16,56+	172.83±129.82	171.74±128.74	236.55±438.22	130.62±157.73	0.099
CD3+/HLA-DR+	209.23±144.29	211.67±147.26	309.19±321.31	277.79±288.93	0.306
CD8+/HLA-DR+	120.40±106.26	127.26±115.28	196.33±286.75	174.88±241.95	0.307
CD8+/CD38+	249.80±200.64	359.54±284.81*	251.90±241.90	308.88±258.39*	0.089
CD19	185.28±130.85	196.36±121.81	183.10±124.05	192.14±120.35	0.856
CD4+/CD45RA	290.5±215.98	360.79±297.03*,†	313.81±254.29	297.81±218.13	0.032
CD4+/CD45RO	531.8±283.86	587.15±306.58*	502.98±217.42	520.98±251.22	0.272

* significantly different from baseline (p<0.05)
† significantly different from control group (p<0.05)
[a] P value comparing both groups

The ratio of CD4+ to CD8+ was not significant in group comparisons (p=0.979).

Natural killer cell numbers increased after three months in the supplemented group (p= 0.026) but were not statistically significantly altered on comparing the two groups, at the end of study (p= 0.509).

The number of cytotoxic T-lymphocytes increased in both groups significantly but there was no significant difference in the group comparison (p=0.089).

The intake of micronutrients had an effect on the shift from CD4+/CD45RO "memory" cells to CD4+/CD45RA+ "naïve" T-cells. The supplemented group showed a significant increase in CD4+/CD45RA+ subsets compared to the control group (p=0.032). Compared to baseline the change in naïve T-cells in the supplemented group was also statistically significant (p=0.026) (Figure 1).

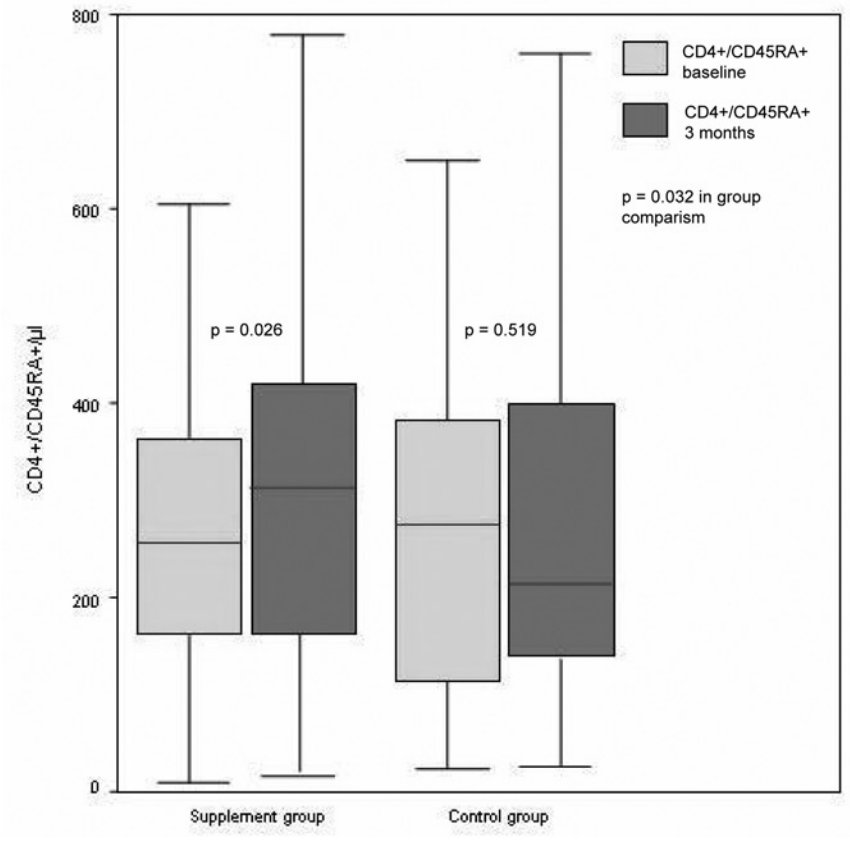

Figure 1: Box plots comparing (p=0.032) CD4+/CD45RA+ T-lymphocytes in a group taking micronutrient supplementation (n=39) and a control group (n=42). Values were measured before and after three months of micronutrient supplementation in addition to standard nursing home meals. The horizontal line through the boxes denotes the median, and the variance bars mark the 90th and the 10th percentile.

No significant difference was seen with respect to B-lymphocytes between groups (p=0.856).

Lymphocyte activity

Both groups showed a significant increase in activated lymphocyte numbers (p<0.001), but there was no significant difference (p=0.095) between groups. No statistical significance was observed after evaluating the number of IL-2 receptors per 1000 cells comparing placebo and supplement, although an increase could be seen within both groups after three months (p<0.001). This difference correlates with changes in IL2 production after stimulation. Both groups showed an IL2 increase after comparing baseline values and values after three months, whereas no statistical significance could be observed between the two groups (p=0.102) (Figure 2).

Antibody titer

Evaluation of antibody titers of the three different influenza strains (Table IV) showed no relationship to micronutrient supplementation. Titers to the B/Yamanashi/166/98 strain were significantly increased in the control group whereas no changes were observed regarding A/New Caledonia/20/99. The antibody titer of the A/Panama/2007/99 strain could not be evaluated because of highly different baseline values between the two groups.

Cholesterol

A significant decrease in cholesterol levels (from 228.72±56.11 to 210.74±52.58, p=0.002), and especially of low-density lipoprotein (LDL) (from 145.75±48.86 to 125.47±41.72, p<0.001), was detected in the group consuming the micronutrients (Figure 3).

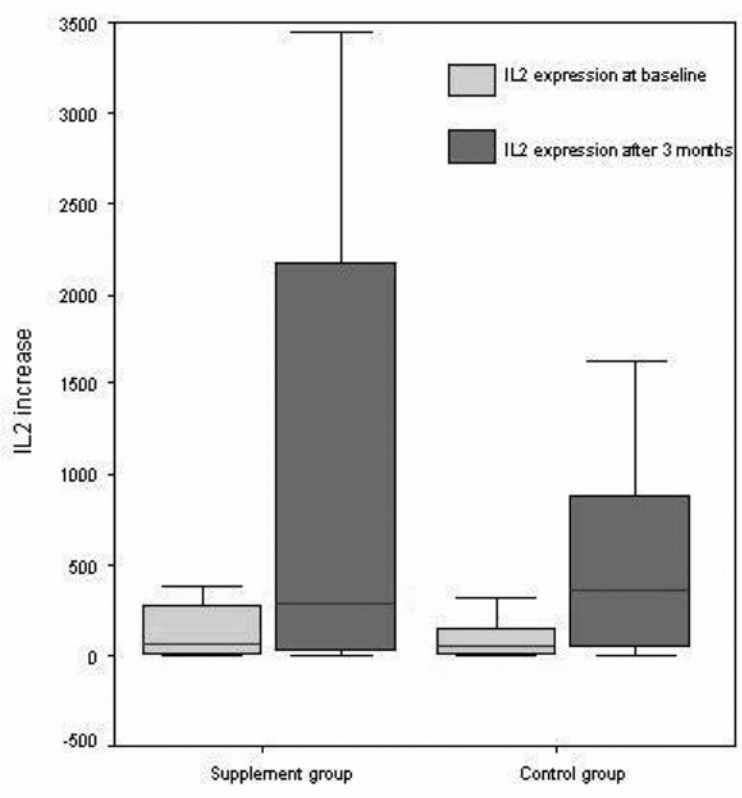

Figure 2: Box plot comparing IL2 mRNA expression in supplement (n=38) and control groups (n=38). Values were assessed at baseline and after three months of supplement consumption compared to GAPDH mRNA expression after lectin stimulation. No significant difference was observed in the supplement group after three months compared to baseline or after comparing the supplement group with the control group (p=0.102). The line through the box denotes the median, the variance lines mark the 90th and the 10th percentiles.

Table IV: Antibody titers of three different influenza strains after 90 days of continuous micronutrient supplementation. Influenza vaccination occurred 8 weeks after start of study (Mean±SD)

	Supplement		Control	
	Baseline	3 months	Baseline	3 months
A/New Caledonia/20/99	13.68±16.67	177.11±204.34	20.00±32.86	201.95±288.68
A/Panama/2007/99	13.16±19.74	130.26±153.28	58.0596.00	284.88±339.14
B/Yamanashi/166/98	77.11±83.95	162.63±146.98*	79.51±102.42	242.44±249.33

* significantly different from control group (p=0.042)

Discussion

This study evaluated the effect of a complex micronutrient supplement on various immune parameters in elderly people. Several significant differences were observed between groups. Its outcome is strengthened by the fact that the baseline characteristics of the subjects did not reveal signs of severe undernourishment with respect to BMI. It is possible that greater differences in measured parameters may have been observed if a more frail and undernourished population had been studied.

In recent years numerous studies have investigated the age-related changes in immune-responsiveness and have shown that the ability of stem cells to undergo clonal proliferation and to mature in lymphoid tissues decreases with age. Elderly individuals also demonstrate a decreased number of lymphocyte subsets in contrast to younger counterparts [22].

It is well known that an increase in total leukocytes occurs in response to infection and inflammation. Taking the evaluated parameters, including C-reactive protein, blood sedimentation time, and a concomitant medication inventory, as well as body temperature (Table II) in account, there was no evidence that the observed significant increase in leukocyte and lymphocyte numbers in the supplemented group was related to infection.

During the aging process the number of antigen-inexperienced CD45RA+ cells decreases, probably

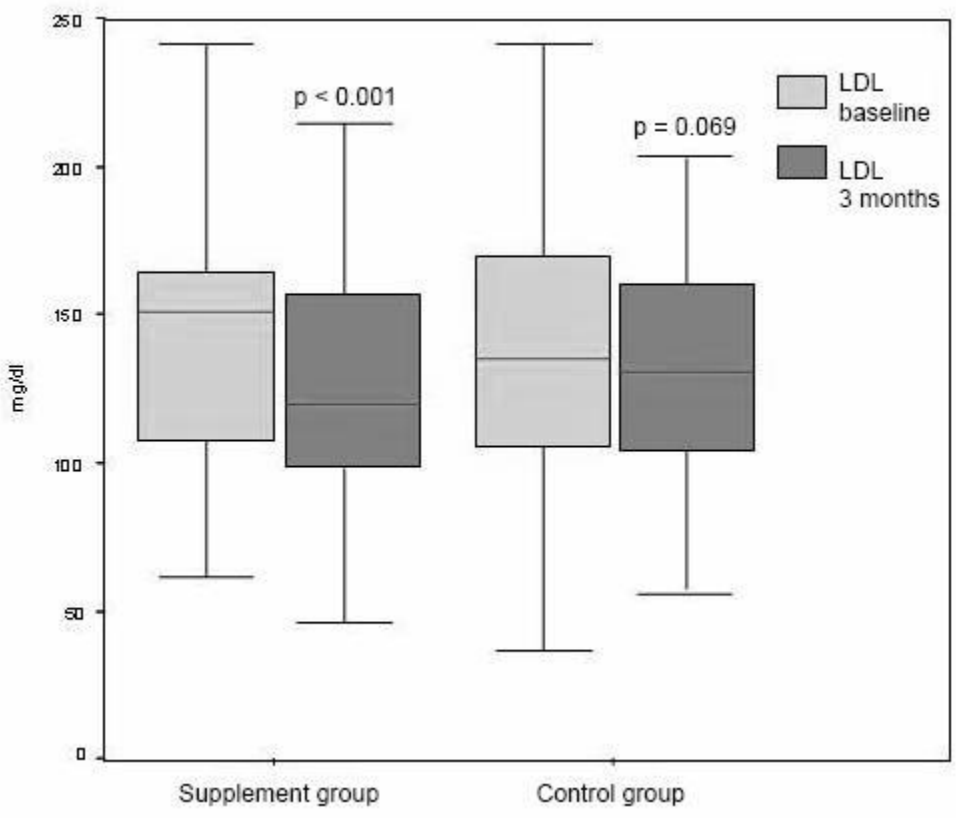

Figure 3: Box plot showing the difference in LDL concentrations in both groups (supplement group: n=38; control group: n=40). The line through the box denotes the median, the variance lines show the 90th and 10th percentiles.

as a result of decreased output of T-cells from the thymus and an increased challenge to antigens [23]. Increased apoptosis of CD45RA+ cells, with activation and accumulation of CD45RO+ T cells, may occur in aged subjects leading to T-cell immune deficiency [24].

In this study we demonstrated that there was a shift from CD45RO+ memory T-cells to CD45RA+ naïve T-cells in the supplemented group (Figure 1). These data imply that the newly derived CD45RA+ T-cells may contribute to the recognition of novel antigens. During the study the total amount of lymphocytes increased, but the number of memory T-cells remained unchanged. We can therefore assume that the higher number of CD45RA+ cells was not derived from a CD45RO+ conversion.

IL2 expression decreases with age, simultaneously with the induction of IL2 mRNA and the expression of IL2 receptors [25]. Naïve T-cells are known to produce higher levels of IL2 [26], which regulates the growth and function of various cells involved in cellular and humoral immunity. An increase in CD45RA+ naïve T-cells should therefore result in an increased secretion of IL2. This investigation showed neither a typical age-related decrease in IL2 nor a significant elevation of IL2 mRNA.

Insufficient antibody formation to influenza antigens has health consequences for the elderly [27]. Immunocompetence is usually influenced by several factors, especially the number of T-cells, their subset composition and cytokine production. All these factors are related to the nutritional status of the elderly [22].

Chandra [17] showed a positive effect of micronutrient supplementation on the immune response to vaccination with respect to the number of T cells and NK cells, lymphocyte response to phytohemagglutinin, IL2 production, IL2 receptor release, NK cell activity, and antibody response to influenza vaccine. But questions regarding validity of this research have been posed [28]. Our results did not show a positive effect on the humoral immune response to vaccination. The control group showed a positive significant difference compared to the supplemented group in antibody titers against the Yamanashi strain, but there was no significant difference between groups in antibody titers against the Caledonia strain. A comparison between the two groups, especially con-

cerning the Panama strain, could not be shown due to the extreme divergence of baseline titer values. Variations in antibody response to influenza virus vaccination are reported to be strain-dependent [29] and dependent on a pre-existing immunity to similar strains [30]. It has also been shown, however, that well-nourished elderly people are not as much affected by reduced immune function compared with their malnourished counterparts [31]. The fact that the humoral immune response was not altered in this study by the micronutrient supplement could have been due to unchanged IL2 levels and the unchanged quantity of CD45RO+ T-cells. These findings correspond with those of Remarque *et al.* [32] who suggest a positive correlation between antibody response to influenza vaccination and CD45RO+ cells in the healthy elderly.

In the study conducted by Chandra [17], involving a multivitamin/mineral supplement in healthy, free-living elderly, supplementation improved immune response as defined by increased NK cell number and activity [26]. The hypothesis that a loss of internal NK cell activity contributes to increasing sensitivity to neoplastic and viral illnesses warrants further research [5,7]. Our results show an increase in NK cells in both treatment groups and although there was a higher increase in NK cell numbers in the supplemented group, the difference was not statistically significant.

In addition to decreased lymphocyte proliferation in response to mitogens and antigens, changes in lymphocyte subsets and alteration to cytokine function and lipid metabolism play a role in altered immune responsiveness of the elderly [33,34]. Cholesterol, in particular, is required for proper function and survival of all cells. Huber *et al.* showed that membrane microviscosity correlates positively with membrane cholesterol/phospholipid molar ratios, which were significantly elevated in the elderly [33]. Wick *et al.* focused on the possible role of altered lipid metabolism in immune system aging. LDL receptor uptake is unexpectedly increased in the elderly, but LDL receptor regulation and serum LDL composition itself seem to be normal [34]. The supplemented group in this study showed a significant decrease in cholesterol and LDL levels (Figure 3), suggesting a possible positive effect on membrane fluidity of lymphocytes and monocytes. Unsaturated fatty acids in combination with antioxidants contained in the supplement may have had a positive influence on lipid metabolism [35].

In conclusion, since many age-related diseases are associated with a decreased immune response and inflammatory reactions, micronutrients could be of benefit as immunostimulants. Although this investigation showed positive effects on cellular immunity and decreased cholesterol and LDL levels in elderly people, further research is warranted to confirm if these effects translate into decreased rates of infection in old people and on other age-related conditions, including autoimmune and degenerative diseases [36].

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