

Nutritional Status Predicts Primary Subclasses of T Cells and the Lymphocyte Proliferation Response in Healthy Older Women¹

Roshni R. Molls,* Namanjeet Ahluwalia,^{†**2} Andrea M. Mastro,[‡]
Helen Smiciklas-Wright,[†] and Gordon C. Handte^{††}

*Graduate Program in Nutrition, Department of Nutritional Sciences, The Pennsylvania State University, University Park, PA 16802; [†]Department of Nutritional Sciences, The Pennsylvania State University, University Park, PA 16802; ^{**}Institut National de la Santé et de la Recherche Médicale, INSERM U558, Epidemiology and Public Health, Hôpital Paul De Viguier, Toulouse, France; [‡]Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA 16802; and ^{††}University Health Services, The Pennsylvania State University, University Park, PA 16802

ABSTRACT Aging is often associated with a dysregulation in immune function, particularly in T-cell responses, even in the healthy elderly. Adequate nutrition is important for optimal immune function. The literature on the relation of nutritional status with immune function in the elderly offers mixed findings. Because several nutrients can influence immune response, and there are interactions among nutrients, examining the association of various nutrients measured simultaneously with tests of immune function is important. We examined the association of protein, iron, zinc, vitamin B-12, and folic acid with tests of acquired immunity in healthy older women (76.7 ± 7.0 y; $n = 130$). Discriminant analysis was used to identify the predictive subset of nutrients that could correctly classify subjects into the lowest or highest quartiles (≤ 25 th or > 75 th percentile) on **various immune function tests (T cells and subsets and lymphocyte proliferation in response to culture with mitogens)**. Protein and iron status variables were identified in the predictive subset for all immune tests; in addition, zinc emerged in the predictive model for T cells and their subsets as well as for the proliferation response to concanavalin A. **The probability of correctly classifying women into the lowest or highest quartiles of immune tests by the predictive subset of nutrition variables was high, i.e., 62.8–83.5% for T cells and their subsets, and 79.3–89.7% for the proliferation response to mitogens.** In conclusion, protein, iron, and zinc were significant predictors of immune function in older women. Adequate status of these nutrients may help maintain immunity in older adults. *J. Nutr.* 135: 2644–2650, 2005.

KEY WORDS: • immune function • nutrition • leukocyte subsets • lymphocyte proliferation • aging

Aging is often associated with increased susceptibility to infection and cancer, as well as an increase in morbidity and mortality associated with these conditions (1). **Age-related changes in the immune system, particularly in T-cell responses, are thought to have an important contributory role in these conditions** (2,3). Nutrition is important for immunocompetence (4–7). Specific nutrient deficiencies can, therefore, further aggravate the age-associated dysfunction in immune function and increase the risk of illness (6,8,9).

Most studies in the elderly examined the association of single nutrients with various aspects of immune function. These studies usually compared immune function in nutrient-deficient vs. nutrient-sufficient subjects (6,10–13) and reported, for the most part, declines in some aspect of immune function when nutrient status was impaired. Further, some

improvements in immune function were noted with nutrient supplementation, especially in deficient subjects upon correction of the deficiency state (14).

Only a few studies made a comprehensive examination of the association of multiple nutrients and immunity in healthy, noninstitutionalized elderly (15–18). These studies generally provided limited evidence to support a relation between nutritional status and immunity. There is speculation (19) that some of these studies were conducted with cohorts lacking variability in nutritional status (15–17). Others did not include key nutrients such as protein and iron status that are likely to be suboptimal in older adults and can affect immune response (15). Although multiple nutrients were assessed in these studies, in most cases, the analyses focused on associations between single nutrients with various aspects of immune function. A multivariate approach is important, however, because multiple nutrient deficiencies can coexist (20,21) and nutrient-nutrient interactions can occur and influence immune function (4–6).

Therefore, our interest was to use a multivariate analysis approach to examine simultaneously the relation of several nutrients with immune function in older subjects with varied

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² To whom correspondence should be addressed.
E-mail: ahluwalia.n@chu-toulouse.fr.

nutritional status. Our goal was to identify which nutrients among those likely to affect immune response when examined simultaneously, would predict immune function in a cohort of healthy older women free from inflammation. We focused on protein, iron, zinc, vitamin B-12, and folate because older adults may be at risk for deficiencies of these nutrients (20–22). The statistical approach of discriminant analysis was used to identify a parsimonious predictive subset of laboratory tests of nutrient status, which could correctly classify healthy older women into the lowest or highest quartiles on several tests of acquired immune response such as T cells and subsets, and lymphocyte proliferation in response to culture with mitogens.

SUBJECTS AND METHODS

Subject recruitment

Older women (age > 60 y; $n = 770$) were invited to participate in the study on nutrition and immunity with the assistance of local Agencies on Aging and recruitment from local housing complexes for seniors using flyers, advertisements, and letters describing the study. Women receiving hormone replacement therapy were not included (23). Subjects provided written informed consent for participation and followed protocols approved by the Office for Regulatory Compliance at the Pennsylvania State University. Subjects received an honorarium of \$100 at the completion of the study.

Study protocol

Potential subjects expressing interest in participation ($n = 180$) were first screened by obtaining a verbal medical history to rule out any health conditions or medication use that could affect immune response according to the SENIEUR protocol (24). Of the 156 women who met the medical history screen, 137 provided a blood sample in the morning after fasting and a 15-min period of rest. A certified phlebotomist collected 35 mL of venous blood into 3 types of Vacutainer[®] tubes (Becton Dickinson), i.e., with no anticoagulant, or with EDTA or heparin. Height and weight were recorded using portable, standardized instruments.

Screening based on laboratory tests

Clinical tests of general health and inflammatory status were used to exclude individuals with acute or chronic inflammation or other medical conditions that could affect immune response. A complete blood count (CBC)³ with differential evaluation on a Coulter MAZM analyzer (Beckman Coulter) and clinical chemistry tests (Chem-24 profile) using a Roche Mira Plus random access chemistry analyzer (Roche Boehringer Mannheim) were conducted at the University Health Services Laboratory. Results of these tests were reviewed by the study physician (G.H.) to exclude subjects with infection, inflammation, liver disorders, kidney disorders, and/or bone marrow proliferation disorders.

To assess inflammation, the erythrocyte sedimentation rate (ESR) was measured using the Westergren Method (Dispette72, Ulster Medical Products), serum α -1 acid glycoprotein (AGP) was determined by radial immunodiffusion (Kent Laboratories), and white blood cell (WBC) count was obtained from the CBC. The cutoff values for defining abnormal results were: ESR > 30 mm/h; WBC > $11 \times 10^9/L$; and AGP > 1.4 g/L (25). Subjects presenting elevated levels on 2 of the 3 inflammatory tests were considered to have inflammation and were excluded from further participation ($n = 7$).

Tests of nutritional status and immune function were also carried out using this blood sample ($n = 130$). An additional blood sample

(25 mL) was obtained whenever possible from subjects ($n = 61$) within 1 wk of the first blood draw to improve precision in the estimates for tests of immune function (26).

Assessment of nutritional status

Laboratory tests were performed to determine protein, iron, zinc, vitamin B-12, and folic acid status. Total protein and albumin were determined in serum as part of the clinical chemistry profile. Serum ferritin was assayed by RIA (Diagnostic Products). Serum transferrin receptors (TfR) were assayed using a commercial ELISA (Ramco Laboratories). Hemoglobin, hematocrit, mean corpuscular volume (MCV), and red cell distribution width (RDW) were obtained from the CBC analysis. Serum iron, total iron-binding capacity, and transferrin saturation were determined by the American Medical Laboratories using colorimetric methods. Serum vitamin B-12 and folic acid concentrations were determined by a commercial RIA (ICN Pharmaceuticals). Pooled serum samples from ongoing studies in our laboratory were used as internal controls with each run for serum ferritin, TfR, vitamin B-12 and folic acid assays. Plasma zinc was measured by atomic absorption spectrophotometry.

Subjects with BMI < 20 kg/m², serum total protein < 60 g/L, or serum albumin < 35 g/L ($n = 28$) were considered to have low protein status (25). Individuals with depleted iron stores [serum ferritin < 20 μ g/L; $n = 5$] (27,28); or tissue iron deficiency [serum TfR > 8.5 mg/L; $n = 4$] alone or in conjunction with abnormal tests of iron transport indices [serum iron < 8.95 μ mol/L (500 μ g/L), total iron binding capacity > 62.65 μ mol/L (3500 μ g/L), or transferrin saturation < 16%; $n = 12$]; or evidence of red cell changes [hemoglobin < 120 g/L, or hematocrit < 0.36, or MCV < 80 fL; $n = 12$] were considered to have low iron status ($n = 33$). Subjects who had plasma zinc levels < 10.71 μ mol/L (700 μ g/L) ($n = 7$) were considered to have low zinc status (29). Subjects with serum vitamin B-12 < 184.5 pmol/L (250 ng/L) ($n = 7$), serum folate < 4.53 nmol/L (2 μ g/L) ($n = 2$) (25,30) and/or MCV > 102 fL ($n = 3$) (31) were considered to have low vitamin B-12 or folate status ($n = 12$).

Assessment of immune function

For sample volume considerations, immune function tests were determined using whole blood assays that were validated previously in our laboratory (2).

T cells and subsets. T cells and subsets were estimated in 1 mL of heparinized blood (32) using fluorescently labeled, monoclonal antibodies specific for surface antigens (anti-CD3 recognizes total T-cells; anti-CD4 recognizes T-helper cells, and anti-CD8 recognizes T-cytotoxic cells) by flow cytometry (Beckman Coulter EPICS XL). CBC with differential evaluation was used to compute the absolute numbers of lymphocytes, monocytes, and granulocytes.

Lymphocyte proliferation response (LPR) to stimulation with mitogens. Proliferation of lymphocytes in response to 2 concentrations each of phytohemagglutinin (PHA) and concanavalin A (Con A) was determined by measuring the incorporation of [³H] thymidine (6.7 Ci/mmol) (33,34). PHA-M (L2646) was obtained from Sigma and Con A from ICN Biochemicals. The 2 optimal concentrations assayed for PHA were 5 and 10 mg/L and those for Con A were 12 and 25 mg/L, respectively, based on our previous study (2). The protocol for this assay was described in detail previously (2,26).

Statistical analyses

Statistical analyses were carried out using the Statistical Analysis System version 8.0 (SAS Institute). Among the nutrition variables, logarithmically transformed data were used for serum ferritin because they were consistent with a normal distribution. For immune function variables, only absolute numbers of T cells and subsets were consistent with normality; therefore, logarithmically transformed data were used for proliferation response related variables. Paired *t* tests for immune function variables on the 2 collection days were not significant (data not shown). Therefore, when data collected on 2 d were available, the mean of the values was used.

The immune function variables examined were T cells and subsets

³ Abbreviations used: AGP, serum α -1 acid glycoprotein; CBC, complete blood count; CD3+, total T cells; CD4+, T-helper cells; CD8+, T-cytotoxic cells; Con A, concanavalin A; ESR, erythrocyte sedimentation rate; LPR, lymphocyte proliferation response; MCV, mean corpuscular volume; NK cells, natural killer cells; PHA, phytohemagglutinin; RDW, red cell distribution width; TfR, serum transferrin receptors; WBC, white blood cell.

TABLE 1

Nutrient status of study participants

Variable	n (%)
Protein status ¹	
Inadequate	28 (21.5)
Adequate	102 (78.5)
Iron status ²	
Inadequate	33 (25.4)
Adequate	97 (74.6)
Zinc status ³	
Inadequate	7 (5.4)
Adequate	123 (94.6)
Vitamin B-12 and folate status ⁴	
Inadequate	12 (9.2)
Adequate	118 (90.8)

¹ Protein status was inadequate if BMI <20, serum total protein <60 g/L or serum albumin <35 g/L.

² Iron status was inadequate if there were depleted iron stores (serum ferritin < 20 µg/L); or tissue iron deficiency (TfR > 8.5 mg/L) alone or in conjunction with abnormal iron transport indices [serum iron < 8.95 µmol/L (500 µg/L), total iron binding capacity > 62.65 µmol/L (3500 µg/L), or transferrin saturation < 16%]; or evidence of red cell changes (hemoglobin < 120 g/L, hematocrit < 0.36, or mean cell volume < 80 fL).

³ Zinc status was inadequate if plasma zinc levels < 10.71 µmol/L (700 µg/L).

⁴ B vitamin status was inadequate if serum vitamin B-12 < 184.5 pmol/L (250 ng/L), serum folic acid < 4.53 nmol/L (2 µg/L), or MCV > 102 fL.

(total T cells, T helper cells, T cytotoxic cells), and LPR to PHA (5 and 10 mg/L) and Con A (12 and 25 mg/L). For each immune function variable, groups were formed on the basis of lowest and highest quartiles. Subjects in the lowest quartile had values ≤ 25th percentile, whereas the highest quartile group had values ≥ 75th percentile of the study cohort for the immune function test. Discriminant analysis was then used to identify an optimal predictive subset of nutritional status variables that could correctly classify subjects into the lowest or highest quartile groups for each immune function test, with a high degree of precision and a low misclassification rate. The nutrition status variables included BMI, serum albumin and serum protein for protein status; serum ferritin, serum TfR, transferrin saturation, hemoglobin, hematocrit, MCV, and RDW for evaluation of iron status, plasma zinc, and serum levels of vitamin B-12 and folic acid as well as MCV for vitamin B-12 and folic acid status.

Preliminary tests using PROC UNIVARIATE and PROC DISCRIM indicated that the assumptions of normality and equal covariance matrixes were met, thus warranting use of the linear discriminant function (35). PROC DISCRIM and PROC STEPWISE were then used to carry out the discriminant analysis. For prediction purposes, a parsimonious set of variables was chosen from the nutrition variables by using the following 4 criteria, which is similar to the approach used in our previous study (27): 1) Evidence suggested by the literature. 2) A large and significant "F to enter," which relates to the significance of the F statistic for group differences in a 1-way ANOVA. A large "F to enter" indicates that the means of the 2 groups were far apart relative to the (pooled) within-group variation. The significance level of 0.20 was used based on the results of Costanza and Affifi (36). 3) A larger and significant "F to remove," relating to the separation of the means of the 2 groups after adjusting for the presence of the other dependant variables in the model (27). 4) The order of entry of the variables in a forward stepwise discriminant analysis, whereby variables that were not significant ($P > 0.20$) were excluded from consideration (36).

Variables that ranked highly using these 4 criteria were selected as the approximate model for the predictive subset. A parsimonious subset of variables (final model) was selected from this near model because it is well known that a parsimonious set of variables is likely

to produce a better predictive subset in terms of cross-validation results (37).

More explicitly, the performance of the final discriminant model was evaluated by using the "leave-one-out" procedure of Morrison and Lachenbruch (35,38). This procedure classifies each subject by using a discriminant function computed from the data points from other subjects in the data set, excluding the observation being classified (27). The CROSSLIST and POSTERR options in SAS DISCRIM were used to carry out the "leave-one-out" cross-validation procedure and obtain the associated error rate for misclassification. We acknowledge that in stepwise discriminant analyses, the calculated significance levels do not reflect the actual error rates and by its nature this procedure selects variables that yield maximal discrimination.

Measures of central tendency and dispersion are indicated as means ± SD unless otherwise indicated. $P < 0.05$ was used as the level of significance.

These analyses were repeated using d 1 data only for immune function variables and yielded similar findings on associations of key nutrients with immune function tests, as reported below; therefore these results are not shown.

RESULTS

The study cohort consisted of 130 generally healthy women (76.7 ± 7.0 y) who were free of underlying inflammation based on a combination of laboratory tests. The majority of the subjects were homebound (74%) and required assistance with activities of daily living. Of the women, 58% were normal weight (BMI < 25 kg/m²), 22% were overweight (BMI between 25 and 30 kg/m²), and 20% had BMI > 30 kg/m².

The nutrient status of study participants was assessed on the basis of comprehensive biochemical tests (Table 1). In this cohort of women, suboptimal status of protein and iron was common (Table 1; 22 and 25%, respectively). Impaired status of zinc was noted in 5% of subjects, and that of vitamin B-12 or folic acid was noted in 9% of subjects.

As expected, the lowest and highest quartile groups differed significantly on the in vitro tests of immune function (Table 2). The values for these immune function variables were consistent with those reported previously for generally healthy and well-nourished young and older adults (2,6,10,12).

T cell and subsets. The lowest and highest quartile groups in T cells and T cell subsets did not differ for any of the nutrition variables examined using a univariate approach (ANOVA; data not shown). However, using the multivariate

TABLE 2

Immune function tests in healthy older women by the lowest and highest quartile for each test¹

Variable	Lowest quartile (n = 31)	Highest quartile (n = 31)
T cell subsets, $n \times 10^9/L$		
CD3+	0.73 ± 0.16	2.33 ± 0.66*
CD4+	0.46 ± 0.14	1.54 ± 0.38*
CD8+	0.18 ± 0.07	0.91 ± 0.37*
LPR, Bq/1000 T cells		
PHA		
5 mg/L	8.5 (5.0, 14.4)	177.4 (122.7, 256.4)*
10 mg/L	20.4 (8.5, 48.9)	223.4 (165.6, 301.3)*
ConA		
12 mg/L	10.2 (5.1, 20.4)	137.7 (102.1, 185.8)*
25 mg/L	13.8 (6.3, 30.1)	134.6 (106.9, 169.4)*

¹ Values are means ± SD or geometric means (−1 SD, +1 SD). * Different from the lowest quartile, $P < 0.0001$.

TABLE 3

Discriminant analysis final models and associated error rates for classification of healthy older women into the lowest and highest quartiles for numbers of T cell subsets and the LPR to mitogens

Immune function variable	Variables selected in the final model	Partial R^2	Misclassification rate with the final model ¹
			%
T cells subsets, $n \times 10^9/L$			
CD3+	Protein Serum ferritin MCV Plasma zinc	0.04 0.04 0.01 0.01	37.2
CD4+	BMI Protein Hemoglobin Hematocrit Plasma zinc	0.04 0.02 0.03 0.04 0.04	16.5
CD8+	Albumin Hemoglobin MCV Plasma zinc	0.07 0.09 0.10 0.07	25.2
LPR, Bq/1000 T cells			
PHA (5 mg/L)	Protein Serum ferritin Hemoglobin Hematocrit RDW	0.01 0.03 0.06 0.05 0.01	15.4
PHA (10 mg/L)	Protein TfR Hemoglobin Hematocrit MCV	0.03 0.01 0.04 0.03 0.08	20.7
ConA (12 mg/L)	Protein Serum ferritin Hemoglobin Hematocrit MCV Plasma zinc	0.06 0.04 0.04 0.04 0.16 0.01	10.3
ConA (25 mg/L)	Protein Serum ferritin Hemoglobin Plasma zinc	0.01 0.09 0.08 0.11	15.0

¹ Error rate indicating the probability for subjects to be classified incorrectly into their group (lowest or highest quartile) based on the final discriminant model.

approach of discriminant analysis, protein, iron, and zinc were identified as the final predictive model for total T (CD3+) as well as T-helper (CD4+) and T-cytotoxic (CD8+) cells (Table 3). The misclassification rates based on this predictive subset of nutrients was highest for total T (CD3+) cell number and lowest for T-helper (CD4+) cell number, 37.2 and 16.5%, respectively (Table 3). Thus, the probability of correctly classifying subjects into the lowest or highest quartiles for CD3+, CD4+ and CD8+ by using the discriminant subset based on protein, iron and zinc status variables ranged from 62.8 to 83.5% for CD3+ and CD4+ cells, respectively.

Lymphocyte proliferation upon assay with PHA or Con A. The proliferation response to 5 mg/L PHA differed significantly between the lowest and highest quartile groups for serum ferritin and hemoglobin (Fig. 1). MCV differed between the lowest and highest quartiles of the LPR to 5 mg/L

Con A, whereas the lowest and highest quartiles on the LPR to 25 mg/L Con A had significantly different plasma zinc concentrations (Fig. 2). For proliferation response to culture with PHA, protein and iron were significant predictors in the final discriminant model (Table 3). Using the discriminant subset of these variables, misclassification rates of 15.4 and 20.7% were obtained for proliferation response to 5 and 10 mg/L PHA, respectively. For the LPR to 12 and 25 mg/L Con A, protein, iron, and zinc were identified in the final predictive models, and provided misclassification rates of 10.3 and 15%, respectively. Therefore, the probability of correctly classifying subjects into highest or lowest quartiles on variables related to LPR to PHA or Con A, using discriminant function models based on variables defining protein, iron, and zinc status, was high and ranged between 79.3 and 89.7%.

DISCUSSION

Nutrition is important for maintaining immune function in the elderly (20). Most studies reported associations of single nutrients with various aspects of immune function in older adults (6,10,17,39–41). The few studies that examined multiple nutrients simultaneously and related nutritional status to immune function had equivocal findings (15,16).

Our approach was unique in that we employed multivariate analysis to determine the relation between various nutrients and immune function in older women with varied nutritional

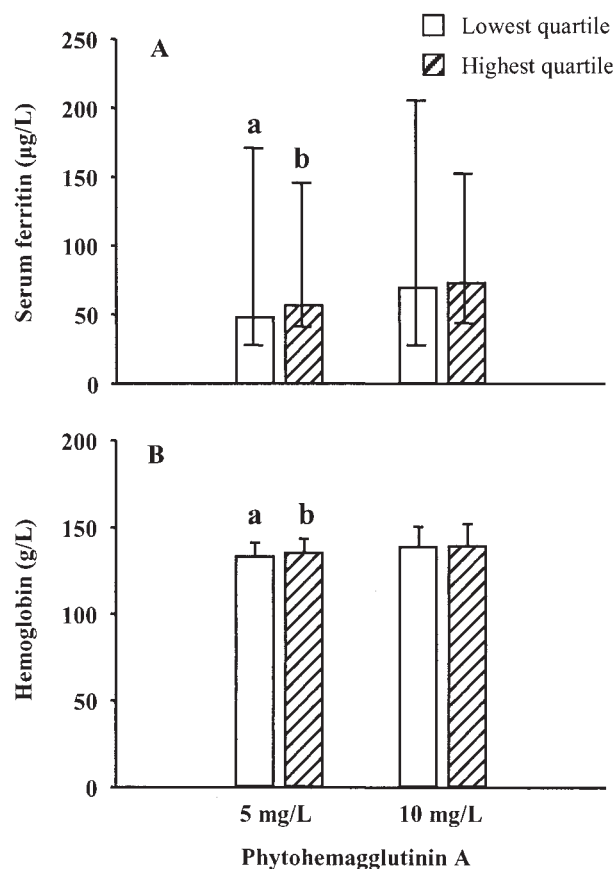


FIGURE 1 Serum ferritin (Panel A) and hemoglobin (Panel B) of healthy older women in the lowest and highest quartiles of lymphocyte proliferation response to PHA. Bars indicate geometric mean \pm SD for serum ferritin and mean \pm SD for hemoglobin, $n = 31$ per quartile group. Means with different letters differ, $P < 0.05$.

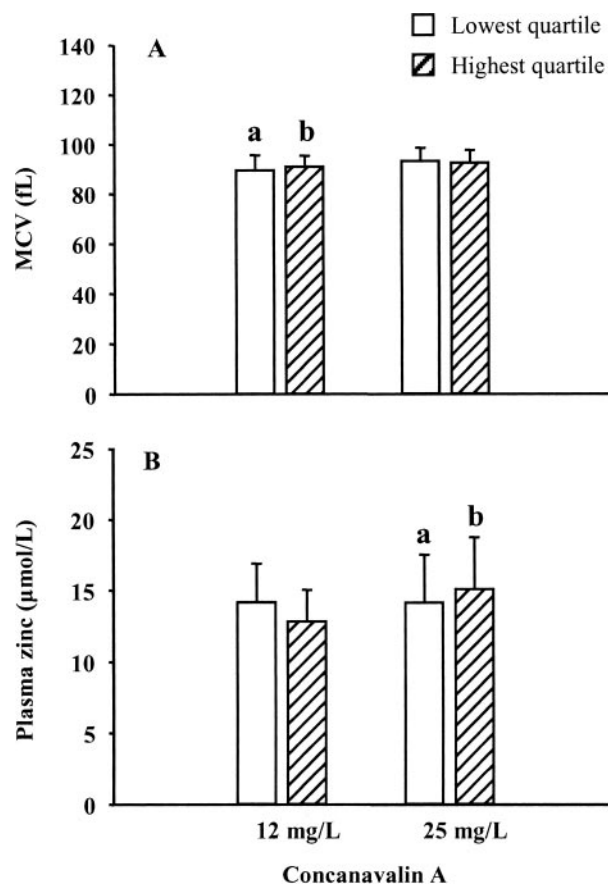


FIGURE 2 Mean cell volume (*Panel A*) and plasma zinc (*Panel B*) of healthy older women in the lowest and highest quartile groups for lymphocyte proliferation response to ConA. Bars indicate mean \pm SD, $n = 31$ per quartile group. Means with different letters differ, $P < 0.05$.

status. Specifically, we identified a subset of nutrients that could categorize healthy older women (>60 y) into the lowest or highest quartile on various tests of acquired immune function by using discriminant analysis. This approach was used because it not only helps identify a parsimonious predictive discriminant model, but also allows the testing of that model in terms of its cross-validation ability and yields the associated misclassification or error rates for classification of subjects into their groups (in our case, the lowest and highest quartiles for each immune function variable). It is noteworthy that 3 of the 5 nutrients examined in the study, i.e., protein, iron, and zinc, emerged as significant predictors of many immune function variables examined in the present study. Furthermore, the probability of classification of subjects accurately into the lowest or highest quartile groups, based on the predictive models consisting of nutritional status variables identified by discriminant analysis, was high, ranging from 62.8 (for CD3+ cell number) to 89% (for LPR to Con A). Thus, the discriminant models based on iron, protein, and zinc status variables performed well in predicting correct classification of subjects into the lowest or highest quartile groups. Factors other than nutrition such as intrinsic biological factors, as well as external factors such as stress and exercise, could account for the remaining unexplained variation (42).

The emergence of key nutrients such as protein, iron, and zinc in the final predictive discriminant models for T cell and their subsets as well as LPR to mitogens is biologically grounded; in fact, these nutrients were shown to predict im-

mune function in several single-nutrient studies (6,10,43–46). Adequate protein status is important for determining lymphocyte counts, lymphocyte proliferation, and antibody responses (6,22). Iron plays important roles in immune function because it is necessary for iron-dependent enzymes such as ribonucleotide reductase and for activation of protein kinase C and hydrolysis of cell-membrane phospholipids that are important in signal transduction, thereby affecting T cell proliferation and other functions (5). Zinc influences the activity of several enzymes important for replication and transcription, such as DNA polymerase and thymidine kinase. It is also a crucial determinant for lymphocyte activation and necessary for optimal activity of phospholipase C and immune mediators such as thymulin. Zinc also affects phosphorylation of protein kinase C and is a major intracellular regulator of lymphocyte apoptosis (7,47).

Interestingly, vitamin B-12 and folic acid, which are important in cell replication and immune response (11,48) because of their function as coenzymes for DNA/nucleic acid synthesis (49), did not appear in the final predictive subset. In the present analyses, subjects with either low vitamin B-12 ($n = 10$) or low folic acid ($n = 2$) status were considered together in 1 common category because of the small number of subjects with inadequate folic acid status, and because both of these vitamins can affect immune function due to their role in cell replication. We reanalyzed the data after excluding subjects with impaired folic acid status ($n = 2$) and after considering B-12 status as an independent variable by itself in the prediction models; however, B-12 status did not appear in the final predictive subset.

We speculate that this lack of association of these B vitamins could be due to less varied status in the study cohort for these nutrients. It is important to indicate, however, that only 5.4% of study cohort had suboptimal zinc status, yet zinc emerged in the final predictive models for T-cell numbers and proliferation response variables. Thus the lack of association of vitamin B-12 and folic acid with immune function tests in the current study cannot be fully explained by the lower prevalence of suboptimal status of these nutrients in the study cohort. It is also possible that these associations were not captured by serum levels of these B vitamins; future studies should, therefore, also examine RBC folate levels or other metabolites of these vitamins.

The contrast of our findings to those reported in the few previous studies involving simultaneous examination of multiple nutrients and tests of immune function in older adults should be noted (15–18). Only 2 of those studies examined the immune function tests conducted in the present study (15–17); for the most part, they did not find any association between nutrition status and immune function tests. The differences across the study findings may be related to the differences in study cohorts in terms of variability in nutrient status, nutrients examined across studies, and in the laboratory techniques used to assess immune function such as use of whole blood vs. isolated mononuclear cells, or differences in mitogens used to stimulate lymphocytes in the proliferation assays.

Despite the fact that nutrient-nutrient interactions can occur, and that multiple nutrient deficiencies often coexist in the elderly, studies to date using multivariate approaches such as regression or discriminant analysis to relate several nutrients with immune function tests have been limited (18). In a cross-sectional study, Payette and colleagues (18) determined the contribution of nutrition factors (status of several nutrients namely protein, iron, zinc, folic acid, vitamin A, and vitamin C, as well as plasma fatty acids) to cytolytic activity of natural

killer (NK) cells and interleukin-2 production. Interestingly, in this multivariate analysis, measures of nutrition status were not associated with NK cell cytotoxicity. The authors speculated that the lack of association was related to the large variability in this index of immune function as well as the fact that few elderly subjects in the study had biochemical evidence of nutrient deficiency except for zinc status. Details on exact prevalence of nutrient deficiencies, however, were not provided. Thus, our study contributes to the limited literature on the contribution of nutritional status to immunologic function in older adults using multivariate analyses and suggests that nutrition factors are important predictors of the phenotypic profile of primary subclasses of T cells and lymphocyte proliferation response upon culture with mitogens in a cohort of elderly with variable nutritional status. There is a need to examine whether this finding holds true for responsiveness and activities of B cells, NK cells, neutrophils, and monocytes/macrophages. The strengths of the current study include the use of participants with a wider range of nutritional status than that in previous studies; comprehensive assessment of nutrient status and immune function tests; and the use of a multivariate approach for data analysis whereby the contributions of several nutrients to immune function were examined simultaneously.

In summary, the findings from the current multivariate analysis relating multiple nutrients simultaneously with immune function variables show the importance of certain nutrients (protein, iron, and zinc) in predicting immune function in healthy older women. However, because of the cross-sectional design, these findings represent associations only and do not establish causal relations. Although there are some indications that the LPR to mitogens is associated with increased mortality (3,50), it would be important in future investigations to also examine the proportions of naïve and memory T cells, B cells, NK cells, phagocytosis and bactericidal function, cytokine production, and in vivo tests of immune function such as the delayed type hypersensitivity skin testing, which were beyond the scope of the present study because of blood volume and cost constraints.

In conclusion, our study supports the notion that key nutrients such as protein, iron, and zinc can predict immune function in older adults. Therefore, it is important that older adults maintain adequate nutritional status overall and for these key nutrients in particular. Further studies are required to elucidate the contribution of nutrition factors to immunologic changes and related diseases in the elderly.

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LITERATURE CITED

1. Makinodan, T. (1995) Patterns of age-related immunologic changes. *Nutr. Rev.* 53: S27-S34.
2. Krause, D., Mastro, A. M., Handte, G., Smicklas-Wright, H., Miles, M. P.

- & Ahluwalia, N. (1999) Immune function did not decline with aging in apparently healthy, well-nourished women. *Mech. Ageing Dev.* 112: 43-57.
3. Murasko, D. M., Weiner, P. & Kaye, D. (1987) Decline in mitogen induced proliferation of lymphocytes with increasing age. *Clin. Exp. Immunol.* 70: 440-448.
4. Beisel, W. R., Edelman, R., Nauss, K. & Suskind, R. (1981) Single-nutrient effects on immunologic function. *J. Am. Med. Assoc.* 245: 53-58.
5. Kuvibidila, S. & Baliga, B. S. (2002) Role of iron in immunity and infection. In: *Nutrition and Immune Function* (Calder, P. C., Field, C. J. & Gill, H. S., eds.), pp. 209-228. CAB International, Oxfordshire, UK.
6. Lesourd, B. (1995) Protein undernutrition as the major cause of decreased immune function in the elderly: clinical and functional implications. *Nutr. Rev.* 53: S86-S94.
7. Shankar, A. H. & Prasad, A. S. (1998) Zinc and immune function: the biological basis of altered resistance to infection. *Am. J. Clin. Nutr.* 68: 447S-463S.
8. Girodon, F., Galan, P., Monget, A. L., Boutron-Ruault, M. C., Brunet-Lecomte, P., Preziosi, P., Arnaud, J., Manuguerra, J. C. & Herchberg, S. (1999) Impact of trace elements and vitamin supplementation on immunity and infections in institutionalized elderly patients: a randomized controlled trial. *MIN. VIT. AOX. geriatric network. Arch. Intern. Med.* 159: 748-754.
9. High, K. P. (2001) Nutritional strategies to boost immunity and prevent infection in elderly individuals. *Clin. Infect. Dis.* 33: 1892-900.
10. Ahluwalia, N., Sun, J., Krause, D., Mastro, A. & Handte, G. (2004) Immune function is impaired in iron-deficient homebound older women. *Am. J. Clin. Nutr.* 79: 516-521.
11. Fata, F. T., Herzlich, B. C., Schiffman, G. & Ast, A. L. (1996) Impaired antibody responses to pneumococcal polysaccharide in elderly patients with low serum vitamin B-12 levels. *Ann. Intern. Med.* 124: 299-304.
12. Mazari, L. & Lesourd, B. M. (1998) Nutritional influences on immune response in healthy aged persons. *Mech. Ageing Dev.* 104: 25-40.
13. Meydani, S. N., Barklund, M. P., Liu, S., Meydani, M., Miller, R. A., Cannon, J. G., Morrow, F. D., Rocklin, R. & Blumberg, J. B. (1990) Vitamin E supplementation enhances cell-mediated immunity in healthy elderly subjects. *Am. J. Clin. Nutr.* 52: 557-563.
14. Prasad, A. S., Fitzgerald, J. T., Hess, J. W., Kaplan, J., Pelen, F. & Dardenne, M. (1993) Zinc deficiency in elderly patients. *Nutrition* 9: 218-224.
15. Gardner, E. M., Bernstein, E. D., Popoff, K. A., Abrutyn, E., Gross, P. & Murasko, D. M. (2000) Immune response to influenza vaccine in healthy elderly: lack of association with plasma beta-carotene, retinol, alpha-tocopherol, or zinc. *Mech. Ageing Dev.* 117: 29-45.
16. Goodwin, J. S. & Garry, P. J. (1983) Relationship between megadose vitamin supplementation and immunological function in a healthy elderly population. *Clin. Exp. Immunol.* 51: 647-653.
17. Goodwin, J. S. & Garry, P. J. (1988) Lack of correlation between indices of nutritional status and immunologic function in elderly humans. *J. Gerontol.* 43: M46-M49.
18. Payette, H., Rola-Pleszczynski, M. & Ghadirian, P. (1990) Nutrition factors in relation to cellular and regulatory immune variables in a free-living elderly population. *Am. J. Clin. Nutr.* 52: 927-932.
19. Bogden, J. D. & Louria, D. B. (1997) Micronutrients and immunity in older people. In: *Preventive Nutrition: The Comprehensive Guide for Health Professionals* (Bendich, A. & Deckelbaum, R. J., eds.), pp. 317-336. Humana Press, Totowa, NJ.
20. Lesourd, B. M., Mazari, L. & Ferry, M. (1998) The role of nutrition in immunity in the aged. *Nutr. Rev.* 56: S113-S125.
21. Tucker, K. (1995) Micronutrient status and aging. *Nutr. Rev.* 53: S9-S15.
22. Chandra, R. K. (2002) Nutrition and the immune system from birth to old age. *Eur. J. Clin. Nutr.* 56: S73-S76.
23. Porter, V. R., Greendale, G. A., Schocken, M., Zhu, X. & Effros, R. B. (2001) Immune effects of hormone replacement therapy in post-menopausal women. *Exp. Gerontol.* 36: 311-326.
24. Lighthart, G. J., Corberand, J. X., Fournier, C., Galanaud, P., Hijmans, W., Kennes, B., Muller-Hermelink, H. K. & Steinmann, G. G. (1984) Admission criteria for immunogerontological studies in man: the SENIEUR protocol. *Mech. Ageing. Dev.* 28: 47-55.
25. Jacobs, D. S., DeMott, W. R. & Oxley, D. K. (2001) *Laboratory Test Handbook*. Lexi-Comp Inc., Hudson, OH.
26. Molls, R. R., Ahluwalia, N., Fick, T., Mastro, A. M., Wagstaff, D., Handte, G. & Ball, R. (2003) Inter- and intra-individual variation in tests of cell-mediated immunity in young and old women. *Mech. Ageing Dev.* 124: 619-627.
27. Ahluwalia, N., Lammi-Keefe, C. J., Bendel, R. B., Morse, E. E., Beard, J. L. & Haley, N. R. (1995) Iron deficiency and anemia of chronic disease in elderly women: a discriminant-analysis approach for differentiation. *Am. J. Clin. Nutr.* 61: 590-596.
28. Milman, N., Pederson, N. S. & Visfeldt, J. (1983) Serum ferritin in healthy Danes: relation to marrow haemosiderin iron stores. *Dan. Med. Bull.* 30: 115-120.
29. Gibson, R. S. (1990) *Principles of Nutrition Assessment*. Oxford University Press, New York, NY.
30. Carmel, R. (1988) Pernicious anemia: the expected findings of very low serum cobalamin levels, anemia, and macrocytosis are often lacking. *Arch. Intern. Med.* 148: 1712-1714.
31. Hillman, R. S. & Finch, C. A. (1996) *Red Cell Manual*. FA Davis Co., Philadelphia, PA.

32. Mandy, F. F., Bergeron, M. & Minkus, T. (1997) Evolution of leukocyte immunophenotyping as influenced by the HIV/AIDS pandemic: a short history of the development of gating strategies for CD4+ T-cell enumeration. *Cytometry* 30: 157–165.
33. Bloemena, E., Roos, M.T.L., Van Heijst, J.L.A.M., Vosses, J.M.J.J. & Schellekens, P.T.A. (1989) Whole-blood lymphocyte cultures. *J. Immunol. Methods* 122: 161–167.
34. Fletcher, M. A., Baron, G. C., Ashman, G. C., Fisci, M. A. & Klimas, N. G. (1987) Use of whole blood methods in assessment of immune parameters in immunodeficiency states. *Diagn. Clin. Immunol.* 5: 69–81.
35. Morrison, D. F. (1990) *Multivariate Statistical Methods*. McGraw-Hill, New York, NY.
36. Costanza, M.C. & Affifi, A. A. (1979) Comparison of stopping rules in forward stepwise discriminant analysis. *J. Am. Stat. Assoc.* 74: 777–785.
37. Hand D. J. (1981) *Discrimination and Classification*. John Wiley & Sons, New York, NY.
38. Lachenbruch, P. A. (1975) *Discriminant Analysis*. Hafner Press, New York, NY.
39. Buzina-Suboticanec, K., Buzina, R., Stavljenic, A., Farley, T.M.M., Haller, J., Bergman-Markovic, B. & Gorajscan, M. (1998) Ageing, nutritional status and immune response. *Int. J. Vitam. Nutr. Res.* 68: 133–141.
40. Fülöp, T., Wagner, R. J., Khalil, A., Weber, J., Trottier, L. & Payette, H. (1999) Relationship between the response to influenza vaccination and the nutritional status in institutionalized elderly subjects. *J. Gerontol.* 54A: M59–M64.
41. Meydani, S. N., Ribaya-Mercado, J. D., Russell, R. M., Sahyoun, N., Morrow, F. D. & Gershoff, S. N. (1991) Vitamin B₆ deficiency impairs interleukin 2 production and lymphocyte proliferation in elderly adults. *Am. J. Clin. Nutr.* 53: 1275–1280.
42. Mastro, A. M. & Bonneau, R. H. (2005) Exercise and immunity. In: *The Endocrine System in Sports and Exercise (IOC Encyclopedia)* (Kraemer, W. J. & Rogol, A. D., eds.), pp. 368–390. Blackwell Publishing, Oxford, UK.
43. Bogden, J. D., Oleske, J. M., Lavenhar, M. A., Munves, E. M., Kemp, F. W., Bruening, K. S., Holding, K. J., Denny, T. N., Guarino, M. A., et al. (1990) Effects of one year of supplementation with zinc and other micronutrients on cellular immunity in the elderly. *J. Am. Coll. Nutr.* 9: 214–225.
44. Chandra, R. K., Gupta, S. & Singh, H. (1982) Inducer and suppressor T cell subsets in protein-energy malnutrition. Analysis by monoclonal antibodies. *Nutr. Res.* 2: 21–26.
45. Duchateau, J., Delepesse, G., Vrijens, R. & Collet, H. (1981) Beneficial effects of oral zinc supplementation on the immune response of old people. *Am. J. Med.* 70: 1001–1004.
46. Fortes, C., Forastiere, F., Agabiti, N., Fano, V., Pacifici, R., Virgili, F., Piras, G., Guidi, L., Bartoloni, C., et al. (1998) The effect of zinc and vitamin A supplementation on immune response in an older population. *J. Am. Geriatr. Soc.* 46: 19–26.
47. Fraker, P. J. & King, L. E. (2004) Reprogramming of the immune system during zinc deficiency. *Annu. Rev. Nutr.* 24: 277–98.
48. Gross, R. L., Reid, J. V., Newberne, P. M., Burgess, B., Marston, R. & Hift, W. (1975) Depressed cell-mediated immunity in megaloblastic anemia due to folic acid deficiency. *Am. J. Clin. Nutr.* 28: 225–232.
49. Myrvik, Q. N. (1999) Immunology and nutrition. In: *Modern Nutrition in Health and Disease* (Shils, M. E., Olson, J. A. & Shike, M., eds.), pp. 623–662. Lippincott Williams and Wilkins, Philadelphia, PA.
50. Wayne, S. J., Rhyne, R. L., Garry, P. J. & Goodwin, J. S. (1990) Cell-mediated immunity as a predictor of morbidity and mortality in subjects over 60. *J. Gerontol.* 45: M45–M48.