



EFFECTS OF NUTRITIONAL INTERVENTION ON IMMUNE MARKERS IN MALNOURISHED ELDERLY

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Abstract: *Introduction:* Both malnutrition and advanced age are known to negatively impact the immune system. The aim of this exploratory randomized controlled trial was to study the effects of a composite nutritional intervention on immune markers, endocrine markers and a selection of micronutrients in malnourished ill elderly patients. *Patients and methods:* Malnourished elderly patients (> 60 yrs) newly admitted to the departments of general internal medicine of a university medical center were randomised to receive either usual care plus a multi-component nutritional intervention (energy and protein enriched diet, comprising oral nutritional support, calcium-vitamin D supplement, telephone counselling by a dietitian) for three months post-discharge or usual care alone. Immune markers (interleukins, complement, C-reactive protein, albumin, TNF- α), endocrine markers (growth factors) and micronutrients (iron, ferritin, vitamin A, E and D), were measured at baseline and three months following hospital discharge. *Results:* In the parent study 210 patients were included, 105 in each group. This study is a subanalysis of 89 patients (46 patients in the intervention group and 43 in the control group) of whom both baseline and final measurement of immune markers, endocrine markers and micronutrients were available. This selection of patients appeared to be in a better health status compared to the total group. At baseline, most of the analysed immune markers, endocrine markers and micronutrients showed values within the normal range, with no statistically significant differences between intervention group and control group. Most immune markers, endocrine markers and micronutrients tended to improved over time, without statistically significant differences between groups, except for vitamin D ($p=0.008$), confirming the supplementation of vitamin D in the intervention group. *Conclusion:* A three months nutritional intervention in malnourished ill elderly patients had no measurable additional influence on measured immune markers, endocrine markers and selected micronutrients. The improved outcomes were presumably caused by patients' improved health status during time.

Key words: Malnutrition, immune markers, elderly, nutritional intervention.

Introduction

Malnutrition is of major concern in elderly hospitalized patients as it negatively impacts patients' health and as a consequence has a negative impact on clinical outcome (1, 2). A recent nutritional intervention study (three months of oral nutritional supplements (ONS), additional calcium/vitamin D supplementation and dietetic counselling) showed weight gain and a decrease in functional impairment and falls in

malnourished ill elderly patients (3, 4). These effects could be explained by effects of energy and protein, leading to a positive balance between protein synthesis and protein breakdown (5) and/or vitamin D supplementation, leading to improvement of muscle strength and function (6). However, also restoration of the improved immune system could possibly have affected the recovery of the supplemented group.

Both malnutrition and advanced age are known to negatively impact the immune system. Malnutrition per se affects nearly all aspects of the immune defence system, but especially impairs cell mediated immunity and resistance to infection (7).

In the elderly, many alterations of both innate and adaptive immunity have been described.

Although the emphasis of most research on immunosenescence has been on T cells, there is an increasing realization that the subtle changes seen in parameters of innate immunity, including the acquisition of some characteristics of innate immunity by T cells themselves (8-10), may have more influence on immunity

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than so far assumed (11-13).

Adequate nutrition is believed to play a role in the maintenance and restoration of impaired immune-competence, even in old age (14-16). Not only an adequate intake of energy and protein play an important role. Also, the correction of certain nutritional deficiencies has been demonstrated to improve the host's immunity, which warrants a place for these nutrients in an adequate diet. However, the optimal intake for a variety of micronutrients, to improve host's immunity, has not been established. To obtain an idea of the possible changes in the immune system in the period recovering from disease and malnutrition, a broad range of (surrogate) immune markers (interleukins, complement, C-reactive protein, albumin, TNF- α), endocrine markers (growth factors), and micronutrients (iron, ferritin, vitamins) are assessed, to explore if these different compartments may explain the enhanced recovery of a malnourished ill elderly population following nutritional intervention. The aim of this exploratory randomized controlled trial was to study the effects of a composite nutritional intervention on immune markers, endocrine markers and a selection of micronutrients in malnourished ill elderly patients. We hypothesize that nutritional intervention will improve immune status in this patient group when measured three months post-discharge.

In addition, data on antibiotic treatment is collected, as patients with a slower recovery of their immune system are expected to be more susceptible to (recurrent) infections, and would thus require more antibiotic prescriptions.

Methods

Design

The current study is an exploratory sub-analysis of a parent study; the study design and primary results have been reported elsewhere (3, 4, 17). The parent study was a randomized controlled trial, comparing usual care plus oral nutritional supplements (ONS), calcium/vitamin D supplementation and dietetic counselling versus usual nutritional care alone in malnourished ill elderly patients, from hospital admission up until three months following discharge (17). In short, patients receiving the nutritional intervention improved in body weight and function(3), and fell less often (4) than those who did not receive the intervention. Adherence to ONS, vitamin D supplementation and dietetic counselling was 80%, 96% and 96%, respectively (3). The current sub-analysis focuses on immune markers, endocrine markers and micronutrients. The study designs are in accordance with the Declaration of Helsinki and were approved by the Medical Ethics Committee (METC) of the VU University Medical Center, Amsterdam.

Patients

All elderly patients (≥ 60 years of age), newly admitted (expected length of hospital stay > 2 days) to the departments of general internal medicine, rheumatology, gastroenterology, dermatology, nephrology, orthopaedics, traumatology and vascular surgery of the VU University Medical Center were screened for malnutrition.

Patients were eligible for the study if they were identified to be malnourished according to the following criteria:

- Body Mass Index (BMI in kg/m^2) ≤ 20 and/or
- $\geq 5\%$ unintentional weight loss (self-reported) in the previous month and/or
- $\geq 10\%$ unintentional weight loss (self-reported) in the previous six months.

Details of the study design have been described extensively elsewhere (17). In short: a computerized random number generator was used to assign patients in blocks of ten to either the control group or the intervention group. Patients allocated to the control group received usual care, i.e. they were given nutritional support only on prescription from their treating physician. Generally, they did not receive post-discharge nutritional support on a standard basis.

Patients allocated to the intervention group received standardized nutritional support from hospitalization up until three months following discharge:

- Energy and protein enriched diet (only during the in-hospital period). Compared with the regular hospital menu, this diet could provide an extra intake of about 750 kcal and 30 g protein per day.
- Two additional servings per day of an oral nutritional supplement (ONS; Nutridrink[®], Nutricia N.V., Zoetermeer, The Netherlands) were offered. This was intended to provide per day an additional 600 kcal, 24 g protein, 176 IU vitamin D3, 364 mg calcium, 492 μg -RE vitamin A, 7.6 mg α -TE vitamin E and 9.6 mg iron, next to other macro- and micronutrients. Two bottles/day provide 40% of the recommended daily allowances of macro- and micronutrients) during the entire study period.
- 400 IU vitamin D3 and 500 mg calcium per day, during the entire study period. In the Netherlands vitamin D is usually given as a combined calcium/vitamin D supplement (Calci-Chew D3[®], Nycomed bv, Hoofddorp, The Netherlands,).
- Telephone counselling by a dietitian was conducted every second week following hospital discharge (six sessions in total). The goal of counselling was to encourage adherence to the prescribed supplements.

The intake of all the prescribed supplements, after discharge from hospital, could provide an additional 600





kcal, 24 g protein, 576 IU vitamin D3, 864 mg calcium, 492 µg-RE vitamin A, 7.6 mg α-TE vitamin E and 9.6 mg iron per day.

Outcome parameters

Immune markers, endocrine markers and micronutrients

Limited production and/or diminished functional capacity of all cellular components of the immune system have been reported, both in malnutrition and in old age. To understand the possible changes in the immune system in the period recovering from disease and malnutrition, this study will explore several aspects of the immune system, endocrine system and micronutrients:

- inflammation; to study recovery from disease (C-reactive Protein, albumin)
- (pro)inflammatory cytokines which are pivotal to the inflammation that is associated with malnutrition (IL-1β, IL-2, IL-6, IL-8, IL-12, IL-17A, IL-22, IFN-γ, TNF-α)
- anti-inflammatory cytokines which inhibit production of inflammatory cytokines (IL-4, IL-10)
- endocrine changes; they occur during the systemic inflammatory response (IGF-1)
- complement components; the availability is compromised during malnutrition (C3 and C3d)
- cytolytic activity of cytotoxic T-cells/NK cells (granzyme B)
- micronutrients; they are supportive to the immune system and frequently deficient during malnutrition. Due to the limited amount of samples and decisions made in collection and storage, only iron, ferritin, 25-OH vitamin D, vitamin A and vitamin E were measured.

From each patient two tubes of blood were collected at both baseline and three months following discharge. The blood was centrifuged to remove cellular components and the serum left was frozen until analysis.

Details on storage, laboratory and measurements of the different immune markers, endocrine markers and micronutrients are presented in Table 1. Both samples from each patient were analyzed together in one run in order to minimize variation within patients.

Antibiotics

For the purpose on post-discharge recovery of immune function, post-discharge antibiotic use was regarded as a surrogate parameter. Therefore we collected data on post discharge antibiotic treatment starting from one week following hospital discharge. Patients with a slower recovery of their immune system were expected to be more susceptible to (recurrent) infections, and would

thus require more antibiotic prescriptions. Therefore, data on number of antibiotic prescriptions, duration (days), generic name, way of dispense (oral or intravenous) and dosage were collected.

Statistical methods

Mean (SD) or median (IQR) levels at baseline and at three months following discharge were calculated (for normally and non-normally distributed parameters respectively). Differences between the groups were tested by independent t-tests (normally distributed parameters or Mann-Whitney U tests (non-normally distributed parameters). Differences in dichotomous variables were tested by chi-square tests. Delta's express differences between baseline and final measurement.

Interleukin levels were often below detection limit (absolute value zero), resulting in zero or negative delta's. Therefore the delta's for interleukins were categorized into three groups (<0, 0 and >0) and tested by chi-square.

In addition, sub-analyses by paired t-tests were performed excluding patients with baseline interleukin levels below the detection limit (i.e. excluding patients for whom interleukin levels were already optimal).

Sub-analyses were performed to study if age (\leq versus $>$ the median age of 75 y) and degree of malnutrition (severe malnutrition: BMI $<$ 18.5 and/or $>$ 5% unintentional weight loss in the previous months plus $>$ 10% unintentional weight loss in the previous six months, versus less severe malnutrition: all other) influenced the relation with immune function. The statistical analyses were restricted to patients with complete follow-up. Statistical significance was defined as $P \leq 0.05$. Statistical analyses were performed using the SPSS-system for Windows, version 17.0 (SPSS, Chicago, IL, USA).

Results

In total 210 patients were included in the parent study, 105 in each group. For this explorative study on immune markers, endocrine markers and micronutrients, samples of 89 patients (46 patients in the intervention group and 43 patients in the control group) both baseline and final measurement were available. Sixty patients were lost to follow-up, due to death (n=25) or withdrawal (n=35). Another thirty patients were too ill to visit the outpatient clinic of the hospital for the second blood sample collection and in 31 patients logistical problems were responsible for the failure of the second measurement.

Group selection

At baseline, statistically significant differences were observed between patients with both measurements (n=89) versus patients with only a baseline measurement available (n=192) for age (included patients were





Table 1
Details on detection method, sample, storage and laboratory of immune markers, endocrine markers and micronutrients measured

Parameter	Detection method	Dilution factor	Sample	Storage	Details
Complement C3 in $\mu\text{g/ml}$	Assessed by radial immunodiffusion using mono specific polyclonal rabbit antisera	100	Serum	-80°C	3
Complement C3d in $\mu\text{g/ml}$	A supernatant was assessed by rocket electrophoresis. The antiserum used was a monospecific polyclonal goat antibody against C3d, prepared by immunization with purified C3d. A standard with known contents of C3d was included in each assay	100	Serum	-80°C	3
Ratio complement C3d/C3 in $\mu\text{g/ml}$	Ratio = C3d/C3	-	Serum	-80°C	3
IL-2R in pg/ml	ELISAs* were performed according to the manufacturers instructions of Diaclone	5	Serum	-80°C	2
IL-12 in pg/ml	ELISAs* were performed according to the manufacturers instructions of Biolegend	5	Serum	-80°C	2
IL-17A in pg/ml	ELISAs were performed according to the manufacturers instructions of Diaclone	5	Serum	-80°C	2
IL-22 in pg/ml	ELISAs were performed according to the manufacturers instructions of Biolegend	3	Serum	-80°C	2
IL-1 α in pg/ml	ELISAs* were performed according to the manufacturers instructions of Sanquin	3	Serum	-80°C	2
IL-4 in pg/ml	ELISAs* were performed according to the manufacturers instructions of Sanquin	3	Serum	-80°C	2
IL-6 in pg/ml	ELISAs* were performed according to the manufacturers instructions of Sanquin	3	Serum	-80°C	2
IL-8 in pg/ml	ELISAs* were performed according to the manufacturers instructions of Sanquin	3	Serum	-80°C	2
IL-10 in pg/ml	ELISAs* were performed according to the manufacturers instructions of Sanquin	3	Serum	-80°C	2
TNF- α in pg/ml	ELISAs* were performed according to the manufacturers instructions of Sanquin	3	Serum	-80°C	2
Albumin in g/L	Chemically determined on a Modular P analyzer (ACN 760, 11815148 216, Roche Diagnostics, Almere, The Netherlands)(29)	-	Serum	-20°C	1
Iron in $\mu\text{mol/L}$	Analyzed by a colorimetric assay on a Modular P analyser (Roche diagnostics, Mannheim, Germany)	-	Serum	-20°C	1
Ferritin in $\mu\text{g/L}$	Analyzed by an electro chemiluminescence immunoassay on a Modular E analyser (Roche diagnostics, Mannheim, Germany)	-	Serum	-20°C	1
Total iron binding capacity (TYBC) in $\mu\text{mol/L}$	Calculated by transferrine (g/L) * 25 = TYBC ($\mu\text{mol/L}$)	-	Serum	-20°C	1
Iron saturation in %	Iron saturation was calculated by $100 * [\text{Iron } (\mu\text{mol/L}) / \text{TYBC } (\mu\text{mol/L})] = \% \text{ transferrin saturation}$	-	Serum	-20°C	1
IGF-1 in nmol/L	Determined by an enzyme-labelled chemiluminescent immunometric assay on a Immulite 2500 (Siemens medical solutions Diagnostic, USA)	-	Serum	-80°C	1
CRP in mg/L	Analyzed by an automated latex-enhanced immunoturbidimetric assay on a Modular P analyser(30).	-	Plasma	-20°C	1
25-hydroxy vitamin D in nmol/L	Analyzed by radioimmunoassay (Diasorin, Stillwater, MN, USA)	-	Serum	-20°C	1
Vitamin A (retinol) in $\mu\text{mol/L}$	Determined by a modification of a published procedure(31). Briefly, after addition of the internal standard tocol, the plasma samples were de-proteinized with ethanol and extracted with n-hexane. After evaporation of the extract, the residue was dissolved in ethanol and analyzed by isocratic reversed-phase high-performance liquid chromatography. Detection was performed by UV absorption at wavelengths of 325 nm.	4	Plasma	-80°C	Sera were re-frozen at -80°C until analysis 4
Vitamin E (α -tocopherol) in $\mu\text{mol/L}$	Determined by a modification of a published procedure(31). Briefly, after addition of the internal standard tocol, the plasma samples were deproteinized with ethanol and extracted with n-hexane. After evaporation of the extract, the residue was dissolved in ethanol and analyzed by isocratic reversed-phase high-performance liquid chromatography. Detection was performed by UV absorption at wavelengths of 280 nm.	4	Plasma	-80°C	Sera were re-frozen at -80°C until analysis 4

* ELISAs; Enzyme Linked Immuno-Sorbent Assay; 1. Endocrine Laboratory of the VU University Medical Center; 2. Medical Immunology Laboratory of the VU University Medical Center; 3 Medical Immunology Laboratory of the Leiden University Medical Center; 4 Metabolic Laboratory of the VU University Medical Center





younger, $p < 0.001$), gender (included patients were more often male, $p = 0.001$), body weight (included patients had a higher body weight, $p = 0.001$), iron (included patients had higher iron levels, $p = 0.003$), iron saturation (included patients had higher iron saturation percentages, $p = 0.004$) and IL-12 (included patients had higher IL-12 levels, $p = 0.042$).

In contrast to previous published results within the total group of 210 patients (3), in this subgroup of 89 patients no statistically significant improvement between groups for functional limitations $p = 0.538$, 95% CI -0.747 to 0.3926), number of falls ($p = 0.108$, 95% CI -0.384 to 3.811) and body weight ($p = 0.378$, 95% CI -0.341 to 0.131) could be demonstrated following three months of nutritional intervention.

Baseline and clinical characteristics

No statistically significant differences in baseline and clinical characteristics between groups were observed (Table 2). Granzyme B was below detection limits in all patients at both time points, whereas IFN- γ levels were below detection limits in 46 out of a pilot of 50 patients. Decision was made not to analyze IFN- γ in other patients.

Immune markers and endocrine markers

At baseline, immune markers and endocrine markers were within the normal range of reference values for the majority of patients, with no statistically significant differences between the intervention and control group (Table 3). CRP (inflammatory marker) was increased and

albumin (negative phase protein) was decreased, in conformity with the acute hospital admission. Most interleukin levels were below detection limits. Therefore, sub-analysis were performed in which levels were split up in two groups: under or above the median. No statistically significant differences between groups could be demonstrated.

At three months following discharge, several interleukins showed significant differences between baseline measurement and final measurement. These differences were absent when only patients with interleukin levels above zero at baseline were selected, except for IL-1 β in the intervention group ($p = 0.022$). The final measurement at three months showed no differences between groups.

Micronutrients

At baseline, micronutrient levels were within the normal range of reference values for the majority of patients, except for vitamin D (deficient levels) and iron and total iron binding capacity (decreased levels, matching with the observed acute phase response). No statistically significant differences between the two groups were observed (Table 3).

Three months following discharge, there were no statistically significant differences between groups for vitamin A, E and iron, whereby total iron binding capacity had increased to normal values. However, a statistically significant increase in vitamin D level was demonstrated in favour of the patients allocated to the

Table 2
Baseline and clinical characteristics between the intervention group versus the control group

Characteristics	Intervention group (n=46)	Control group (n=43)	P-value
Gender – number of females (%)	17 (37.0)	20 (46.5)	0.407 ^b
Age in yr – mean (\pm SD)	71.6 (8.4)	71.3 (7.8)	0.733 ^a
Home situation – number (%)			0.533 ^b
Living alone	19 (41.3)	21 (48.8)	
Living with partner / family	27 (58.7)	22 (51.2)	
Consulting dietician pre-admission – number (%)	15 (32.6)	7 (16.3)	0.103 ^b
Use of supplemental drinks pre-admission – number (%)	11 (23.9)	10 (23.3)	0.896 ^b
Body weight – mean (\pm SD)	65.5 (14.6)	60.8 (13.4)	0.114 ^a
Men	69.1 (15.6)	66.0 (9.9)	
Women	59.4 (10.3)	54.9 (10.2)	
Body Mass Index in categories – number (%)			0.187 ^b
BMI \leq 20	22 (47.8)	21 (48.8)	
BMI 20 – 25	14 (30.4)	18 (41.9)	
BMI \geq 25	10 (21.7)	4 (9.3)	
Weight change last month – % (\pm SD)	-4.9 (5.4)	-3.5 (7.7)	0.343 ^a
Weight change last six months – % (\pm SD)	-9.7 (7.3)	-8.1 (7.7)	0.359 ^a
Primary medical diagnosis – number (%)			0.396 ^b
Acute	25 (54.3)	20 (46.5)	
Chronic	21 (45.7)	23 (53.5)	
Primary medical diagnosis in categories – number (%)			0.339 ^b
Acute infections	12 (26.1)	4 (9.3)	
Vascular disease	7 (15.2)	9 (20.9)	
Kidney insufficiency	6 (13.0)	5 (11.6)	
Fractures, orthopaedic disorders	3 (6.5)	6 (14.0)	
Malignant neoplasm	3 (6.5)	6 (14.0)	
Chronic bowel disease	7 (15.2)	7 (16.3)	
Diabetes Mellitus, heart failure and other	4 (8.7)	2 (2.7)	
Bleeding in gastrointestinal tract	3 (6.5)	1 (2.3)	
Liver, gall and pancreas insufficiency	1 (2.2)	3 (7.0)	

a. t-test; b. Chi² test



**Table 3**

Differences in immune markers, endocrine markers and micronutrients between hospital admission and three months after discharge in patients in the intervention group versus patients in the control group

Parameter	Reference value*	Intervention group			Control group			Difference in Δ (95% CI)
		Baseline n=46	Final n=46	Difference baseline and final	Baseline n=43	Final n=43	Difference baseline and final	
Complement C3 †	470-800 $\mu\text{g/ml}$	683.6 (149.4)	661.8 (111.8)	0.241	663.9 (150.0)	670.5 (112.6)	0.266	0.463 (-37.0 to 80.7) ^a
Complement C3d †	0-29 $\mu\text{g/ml}$	16.5 (7.4)	11.1 (4.5)	0.238	17.4 (9.3)	13.1 (7.7)	0.238	0.530 (-2.4 to 4.7) ^a
Ratio complement C3d/C3 †	-	0.03 (0.02)	0.02 (0.01)	0.238	0.03 (0.02)	0.02 (0.01)	0.238	0.783 (-0.01 to 0.01) ^a
CRP ‡	< 8.0 mg/L	37.5 (3.0-110.5)	5.0 (2.5-17.3)	0.013	28.5 (8.0-69.0)	2.8 (2.5-19.3)	0.045	0.162 ^c
IL-12 ‡	< 2 pg/ml	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.000	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.000	0.000 ^b
IL-1 α ‡	< 5 pg/ml	0.02 (0.0-0.6)	0.01 (0.0-1.4)	0.000	0.02 (0.0-1.2)	0.01 (0.0-1.5)	0.000	0.567 ^b
TNF- α †	< 10 pg/ml	25.1 (103.1)	19.2 (63.0)	0.000	2.5 (9.0)	8.4 (43.2)	0.000	0.662 ^b
IL-2R ‡	< 6400 pg/ml	4945 (3231-9860)	4190 (2291-7660)	0.241	4177 (2791-6392)	3234 (3232-6284)	0.241	0.661 ^b
IL-17A ‡	< 3 pg/ml	0.0 (0.0-29.0)	0.0 (0.0-29.8)	0.000	0.0 (0.0-35.0)	0.0 (0.0-22.5)	0.000	0.334 ^b
IL-22 ‡	< 10 pg/ml	181.6 (0.0-340.3)	0.0 (0.0-229.5)	0.000	161.6 (0.0-251.7)	0.0 (0.0-231.2)	0.000	0.663 ^b
IL-6 ‡	< 10 pg/ml	5.3 (0.0-18.9)	0.0 (0.0-0.0)	0.964	3.2 (0.0-13.5)	0.0 (0.0-0.0)	0.857	0.889 ^b
IL-8 ‡	< 10 pg/ml	22.4 (16.1-43.1)	18.9 (11.2-22.9)	0.238	22.0 (10.7-47.3)	18.0 (10.6-23.8)	0.238	0.639 ^b
IL-4 ‡	< 1 pg/ml	0.0 (0.0-9.1)	1.9 (0.0-7.2)	0.000	0.0 (0.0-5.9)	0.0 (0.0-2.9)	0.000	0.729 ^b
IL-10 ‡	< 5 pg/ml	0.0 (0.0-1.3)	0.0 (0.0-0.0)	0.000	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.000	0.834 ^b
Albumin †	35-52 g/L	30.9 (6.9)	38.7 (7.3)	0.153	31.4 (5.7)	38.9 (6.8)	0.555	0.968 (-3.3 to 3.4) ^a
Iron ‡	11-32 $\mu\text{mol/L}$	9.0 (5.0-16.0)	11.0 (9.0-16.0)	0.109	7.0 (4.3-12.8)	12.0 (8.3-16.0)	0.083	0.508 ^c
Ferritin ‡	20-375 $\mu\text{g/L}$	325.0 (153-586)	107.0 (56-321)	0.249	290.0 (138-681)	169 (55-391)	0.241	0.929 ^c
Total iron binding capacity ‡	45-90 $\mu\text{mol/L}$	47.0 (35.0-56.0)	61.0 (52.0-70.0)	0.097	46.0 (36.0-58.0)	57.0 (50.0-68.0)	0.513	0.243 ^c
Iron saturation ‡	12-50%	17.0 (12.0-34.0)	20.0 (14.0-26.0)	0.084	14.0 (10.0-27.8)	20.5 (13.0-28.0)	0.534	0.211 ^c
IGF-1 †	6.8-23 nmol/L	11.9 (6.0)	16.4 (8.2)	0.284	11.8 (6.9)	15.9 (7.5)	0.252	0.675 (-4.3 to 2.8) ^a
25-hydroxy vitamin D †	>50 nmol/L	41.7 (20.2)	66.0 (25.0)	0.236	43.6 (22.2)	56.4 (25.5)	0.134	0.008 (2.9 to 19.0) ^a
Vitamin A †	1.2-3.0 $\mu\text{mol/L}$	2.0 (1.0)	2.9 (1.1)	0.241	2.0 (1.0)	2.9 (1.2)	0.243	0.909 (-0.5 to 0.4) ^a
Vitamin E †	20-39 $\mu\text{mol/L}$	25.1 (10.1)	30.3 (10.1)	0.238	27.5 (11.7)	34.3 (11.5)	0.238	0.630 (-2.7 to 4.5) ^a

The deltas between the two measurements were calculated and the statistical differences between the deltas are listed in the final column. † Mean (\pm standard deviation); ‡ Median (inter quartile range 0.25-0.75); * Reference values according to the VU University Medical Center, Amsterdam, the Netherlands. a. t-test; b. Chi2 test; c. Mann-Whitney U test

intervention group ($p=0.008$), which was expected on forehand because of the supplementation of vitamin D to these patients.

Influence of age and degree of malnutrition

Literature describes that older age and more severe malnutrition affect the immune system most. Therefore, sub-analyses have been performed across groups to study whether differences could be demonstrated between younger old patients (≤ 75 year) and older old (> 75 year) patients, or for the most severely malnourished versus the less severely malnourished patients (see methods).

A statistically significant increase for vitamin D was observed in the intervention and control group which was not related to vitamin D levels at baseline. Older old patients improved their vitamin D levels more than younger old patients ($p=0.010$); the severely malnourished patients improved less than the less severely malnourished patients ($p=0.009$).

For all other micronutrients, endocrine markers and immune markers analysed, no statistically significant differences could be demonstrated.

Antibiotics

We collected data on use of antibiotics (starting from one week after hospital discharge) as a surrogate marker

for infections in the follow-up period of this study. For 10 patients out of 210 patients (6 in the intervention group and 4 in the control group) these data were missing. Thirty-four patients out of 200 patients (17%, 14 patients in the intervention group and 20 patients in the control group) used antibiotics following hospital discharge. There were no statistically significant differences between the intervention group and control group regarding the number of patients with antibiotic prescriptions ($p=0.287$), the total number of antibiotic prescriptions ($p=0.286$) and the duration of use ($p=0.226$).

For the subgroup of 80 patients with both measurements available and availability of data on antibiotic use, 22 patients used antibiotics (28%, 7 patients in the intervention group and 15 patients in the control group). Again, there were no statistically significant differences between the intervention group and control group regarding the number of patients with antibiotic prescriptions ($p=0.111$), the total number of antibiotic prescriptions ($p=0.103$) and the duration of use ($p=0.078$).

Discussion

Nutrient deficiencies can cause immuno-suppression and dysregulation of immune responses. From the immune markers, endocrine markers and micronutrients measured in this study, the conclusion can be made that all malnourished ill elderly patients had an improved





health status when measured three months after hospital discharge compared to baseline values upon admission in the hospital. Inflammatory markers decreased, most interleukins decreased and vitamin levels increased.

However, a multicomponent nutritional intervention in this malnourished ill elderly patients group did not demonstrate enhanced recovery, while we hypothesized improved immune status following three months post-discharge nutritional intervention. Next to enhanced recovery, both malnutrition and advanced age are known to negatively impact the immune system. Malnutrition per se affects nearly all aspects of the immune defence system, but especially impairs cell mediated immunity and resistance to infection (7).

Complete blood samples at both time points were available for only 89 out of 210 patients.

As a consequence of the design of the study, patients that could not return to the hospital for the final 3 months measurement were the most severely ill patients and hence excluded from analyses in this exploratory study. Included patients were younger, more often male, had a higher body weight, higher iron levels, higher iron saturation percentages and higher IL-12 levels. The exclusion of the most severely ill patients can possibly explain the absence of change in immune markers, endocrine markers and micronutrients.

Remarkably, most patients had a relatively good starting point. Only part of the inflammatory markers (CRP, TNF- α , IL-22 and IL-8) were above the normal range of reference values and iron was below. Otherwise we saw no measured abnormalities for most measured parameters, which also indicates that there was marginal opportunity for improvement.

In contrast to the parent study, we observed no differences in clinical outcomes between groups in this subset of 89 patients, which may also explain the absence of differences between groups in the measured markers.

Also, we observed no increase in fat mass following the nutritional intervention (3), which may explain the absence of change in immune markers associated with increased fat mass (CRP, IL-6, TNF- α and IGF-1(19)).

Use of antibiotics during the study period may have influenced infection related cytokine (e.g. IL-6 and IL-8) release. Treatment with antibiotics was assessed from one week following hospital discharge to three months following hospital discharge. Therefore it was not possible to evaluate the influence of antibiotics on serum cytokine levels.

In a subgroup of patients with both measurements available and availability of antibiotic usage there were no significant differences between groups on antibiotic usage and number of patients with a primary diagnosis of infections. Therefore it is not likely that antibiotic usage influenced the outcome of the analysis. In a post-hoc analysis, no statistically significant differences

between patients with a decrease in cytokine and CRP levels and the usage of antibiotics could be demonstrated.

This exploratory study was designed to study the effects of 'regular' macronutrients and micronutrients only. It is possible that these malnourished ill elderly patients may have had more specific needs. Future studies could focus on improvement of immune markers by specific immunonutrients described to have an immunomodulatory effect e.g. glutamine, arginine, nucleotides, poly unsaturated fatty acids, probiotics or prebiotics such as fructooligosaccharides or galactooligosaccharides, or a higher dosage of micronutrients (e.g. the vitamins A, B6, B12, C, D, E and folic acid) and trace elements (e.g. iron, copper and selenium) (16, 20-22).

In animal studies, disease (burns, injury, infection) has been associated with depletion of antioxidant vitamins and trace elements. In humans, few studies have described associations between malnutrition, old age and reduced host defence leading to increased susceptibility of infections (1, 18). In contrast to our expectations, this small exploratory study showed that all studied micronutrients (except vitamin D) were within normal ranges at the baseline measurement, which was unexpected because of patients' poor nutritional status. In line with the improving health status of patients, in this study, Levels of micronutrients tended to increase over time in both intervention and control group (although not statistically significant), however, not more in the patients who received the nutritional intervention (accounting for 40% of the recommended daily allowances of micronutrients) than in controls. This may be due to the negative acute phase response (23) which misrepresents levels of a patient's actual micronutrient status. Another explanation may be that the actual body provision of, for example vitamin A and E is stored in the liver whereby serum levels are kept normal until severe deficiency occurs.

At baseline, vitamin D levels were deficient > 50 nmol/l (23, 24). This is in line with previous findings by other studies and a Cochrane review on interventions for preventing falls in elderly people (25, 26). Although the supplied dosage of vitamin D (176 IU by ONS and 400 IU by calcium-vitamin D3 supplementation) was lower than the most recent advice by the Health Council of the Netherlands for elderly people (800 IU/day (24)) serum vitamin D levels increased in both groups, however more in the intervention group than in the control group, most likely due to the extra supplementation to intervention patients (4). Remarkably, vitamin D levels increased more in older old patients than in younger old ones; this was also the case for less severely malnourished patients, irrespective of group assignment and baseline levels. We have no clear explanation for these results.

Although the emphasis of most research on immunosenescence has been on T cells, there is an





increasing insight that the subtle changes seen in parameters of innate immunity, including the acquisition of some characteristics of innate immunity by T cells themselves (8, 9), may have more influence on immunity than so far assumed. Both malnutrition and advanced age are known to negatively impact the immune system. Malnutrition per se affects nearly all aspects of the immune defence system, but especially impairs cell mediated immunity and resistance to infection (7). In the elderly, many alterations of both innate and adaptive immunity have been described (14, 15). In this study, the old and more malnourished subpopulations did not differ in immune markers, endocrine markers and micronutrients from their younger and less malnourished counterparts, except for vitamin D.

Immunosenescence and ageing are accompanied with low grade inflammation. In elderly, significant correlations have been observed between levels of CRP and mortality, even though the absolute levels of CRP are still within the normal range (<8.0 mg/L) (27, 28). The current study shows that acute inflammation (defined as CRP \geq 8.0 mg/L) resolves in most patients over the course of 3 months in both study groups. However, since no comparison was made to a relevant reference group, the current data do not provide insight in the occurrence and relevance of low-grade inflammation at the end of the study period.

Conclusion

Although the nutritional modulation of immune function has attracted much attention recently, predominantly by the use of immunomodulating agents, the presumed immunestimulating role of 'regular' macronutrients and micronutrients, except for vitamin D and E, has received only little attention. This exploratory study on three months nutritional intervention, in a malnourished ill elderly patient group could not demonstrate statistically significant improvement of immune markers, endocrine markers and selected micronutrients. This may be caused by the unintentional selection of healthiest patients, the variety of underlying diseases in patients, the supplied nutritional intervention, the used tests for measuring immune markers and/or the choice that was made to only measure at baseline and three months following hospital discharge.

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