Nutrient status, metabolic health and immune function in healthy and active older people

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Felix Kerlikowsky

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Referent:	Prof. Dr. oec. troph. Andreas Hahn
Korreferent:	Prof. Dr. rer. nat. Karsten Krüger
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Abstract

Background and aim: Nutrition is not only primarily concerned to achieve energy and nutrient needs, but also contributes to long-term health and the prevention of chronic degenerative diseases. The older population is not clearly defined, but is characterised by a phase of life in which the heterogeneity of health status and thus the level of independence increases significantly compared to younger populations. Scientific recommendations on energy and nutrient requirements are not fundamentally different between older people and the general population. However, older people are at greater risk of nutrient deficiencies due to physical, social and cognitive changes. The micronutrients vitamin D, cobalamin, folate and long-chain omega-3 fatty acids (n3 FA) are considered particularly critical in older people. In addition, deficiencies of these micronutrients are thought to increase the incidence and progression of degenerative diseases and to be causal for chronic inflammation in old age. Previous studies investigate nutrient deficiencies in older people in nursing homes or with already impaired health. Limited knowledge exist about nutrient deficiencies in active, independent older people. Therefore, the overall aim of this thesis was to assess and to improve the status of critical micronutrients in physically active and independently living older people and to investigate their impact on a number of health-related biomarkers and inflammation.

Methods: This study was conducted as a 12 week single-centre, two-armed, double-blinded, and randomised clinical trial (RCT). In total, 133 healthy subjects met all the inclusion criteria (≥ 70 years, living home dwelling, and independently) and were included in the study. Exclusion criteria were defined as intake of dietary supplements up to three months before the examination, BMI>35kg/m^2, severe diseases, and intake of immunosuppressant's. The multi-micronutrient supplement (MMN) contained several micronutrients in physiological doses (i.e. 400 µg folic acid, 100 µg cobalamin, 50 µg cholecalciferol, 18 mg tocopherol, 100 µg selenium, 1000 mg EPA/DHA). The status of the following micronutrients was measured: Omega-3 Index (O3I) for relative EPA+DHA levels of total fatty acids in red blood cells, serum 25-hydroxyvitamin D (25-(OH)D), red blood cell folate (RBC folate), and holotranscobalamin (holoTC). In addition, concentrations of methyl malonic acid (MMA) and homocystein (hcy) were measured. Inflammatory biomarkers (i.e. white blood cell count, granulocyte/lymphocyte ratio, platelet count, CRP) were included in an aggregated marker of low-grade inflammation, previously described as the INFLA score.

Results: The prevalence of micronutrient deficiencies was low with 7% having serum concentrations of 25-(OH)D less than 50 nmol/L, 11% having RBC folate concentrations less than 570 nmol/L and 12% having serum concentrations of holoTC less than 50 pmol/L. Sex differences were found only in cobalamin status, where 22% of male and only 8% of female subjects were classified as having a low cobalamin status. A total of 88% failed to achieve a desirable O3I of >8%. However, 63.1% of the total cohort had elevated hcy concentrations. After the intervention, there was a significant increase in all biomarkers of micronutrient status compared to the placebo group, leading to a further improvement in vitamin D, folate, cobalamin and n3 FA status in the intervention group. Hcy concentrations were significantly reduced after 12 weeks of micronutrient supplementation. Depending on age and increase in the O3I, a reduction within the INFLA score was observed. However, the reduction were only significant in subjects aged 80 years and older.

Conclusion: Our findings suggest that physically active and independently living older people with high levels of education, physical activity and health awareness are not necessarily at higher risk of vitamin D, folate and cobalamin deficiency. Specifically, gaps in micronutrient status are also prevalent in healthy and active older people. Regardless of folate and cobalamin status older people benefit from the intervention with a reduction in elevated hcy concentrations. Individual's ≥80 years or with low O3I benefited from MMN supplementation by reducing inflammatory activity.

Keywords: Nutrient status, Micronutrient supplements, Inflammageing

Zusammenfassung

Hintergrund und Ziel: Die Ernährung dient bekanntermaßen nicht nur der Deckung des Energie- und Nährstoffbedarfes, sondern trägt auch zur langfristigen Gesundheit und zur Prävention chronisch-degenerativer Erkrankungen bei. Die Bevölkerungsgruppe der älteren Menschen ist nicht klar definiert, aber durch eine zunehmende Heterogenität des Gesundheitszustandes im Vergleich zu jüngeren Bevölkerungsgruppen gekennzeichnet. Die wissenschaftlichen Empfehlungen zur Energie- und Nährstoffzufuhr unterscheiden sich nicht grundsätzlich zwischen Menschen mittleren Alters und älteren Menschen, deren Heterogenität im Gesundheitszustand bisher unberücksichtig bleibt. Mit zunehmendem Alter steigt jedoch aufgrund altersassoziierter Veränderungen das Risiko einer Unterversorgung, insbesondere bei den Mikronährstoffen: Vitamin D, Vitamin B_{12}, Folat und langkettigen Omega-3-Fettsäuren (n3 FS) die auch in der Allgemeinbevölkerung kritisch sind. Ein Mangel an diesen Mikronährstoffen trägt zur Entstehung und zum Fortschreiten degenerativer Erkrankungen sowie zur Abnahme der Immunfunktion im Alter bei. Frühere Studien haben bereits Nährstoffdefizite bei pflegebedürftigen oder funktionell eingeschränkten älteren Menschen untersucht. Das übergeordnete Ziel dieser Arbeit war es im Gegensatz zu diesen Studien, den Status kritischer Mikronährstoffe bei gesunden, selbstständig lebenden älteren Menschen mit einem hohen Maß an körperlicher Aktivität zu erfassen und zu verbessern, um mögliche günstige Auswirkungen auf eine Reihe von gesundheitsbezogenen Biomarkern und den Entzündungsstatus zu untersuchen.

Methodik: Diese Studie wurde als 12-wöchige, monozentrische, placebokontrollierte, doppelblinde, randomisierte klinische Interventionsstudie durchgeführt. Insgesamt erfüllten 133 Probanden alle Einschlusskriterien (≥ 70 Jahre, selbständig lebend). Als Ausschluss-kriterien galten die Einnahme von Nahrungsergänzungsmitteln bis zu drei Monate vor der Untersuchung, ein BMI >35kg/m^2, schwere Erkrankungen sowie die Einnahme von Immunsuppressiva oder Kortikosteroiden. Das Multi-Mikronährstoffpräparat (MMN) enthielt zahlreiche Mikronährstoffe in physiologischen Dosierungen in Kombination mit einem n3 FS Präparat. Folgende Statusmarker wurden gemessen: Omega-3-Index (O3I), 25-Hydroxyvitamin D (25-(OH)D, Folat in Erythrozyten (RBC-Folat), Holotranscobalamin (holoTC), Methylmalonsäure (MMA) und Homocystein (Hcy). Zusätzlich wurden folgende Entzündungsmarker erhoben: Leukozytenzahl, Granulozyten-Lymphozyten-Verhältnis, Thrombozytenzahl und CRP, die anschließend zu einem inflammatorischen Score (INFLA Score) aggregiert wurden.

Ergebnisse: Die Prävalenz von Mikronährstoffdefiziten war insgesamt gering: 7% der Teilnehmer hatten Serumkonzentrationen von 25-(OH)D unter 50 nmol/L, 11% hatten Konzentrationen an RBC Folat unter 570 nmol/L und 12% hatten Serumkonzentrationen von holoTC unter 50 pmol/L. Geschlechtsspezifische Unterschiede zeigten sich nur beim Vitamin B_{12} Status: 22% der Männer und nur 8% der Frauen wiesen einen niedrigen Vitamin B_{12} Status auf. Insgesamt erreichten 88% nicht einen wünschenswerten O3I von 8-11%. Desweiteren wiesen 63,1% der Probanden erhöhte Hcy-Konzentrationen auf. Nach der Intervention mit dem MMN kam es zu einer signifikanten Verbesserung des Vitamin D-, Folsäure-, Vitamin B_{12}- und n3 FS Status in der Interventionsgruppe. Erhöhte Hcy-Konzentrationen wurden durch die Intervention mit dem MMN signifikant reduziert. Eine Abnahme des INFLA-Scores wurde in Abhängigkeit vom Alter und dem Anstieg des O3I beobachtet.

Schlussfolgerung: Gesunde, selbstständige ältere Menschen sind überwiegend gut versorgt mit den Vitaminen D, Folat und Vitamin B_{12}. Dennoch treten vereinzelt Versorgungslücken auf. Die Versorgung mit n3 FS entsprach der überwiegend unzureichenden Versorgung der Allgemeinbevölkerung. Unabhängig vom Folsäure- und Vitamin B_{12}-Status profitieren ältere Menschen von einer MMN-Supplementierung durch eine Reduktion erhöhter Hcy-Konzentrationen und in Abhängigkeit vom O3I durch eine Reduktion der Entzündungsaktivität.

Schlagwörter: Nährstoffstatus, Multinährstoffsupplemente, Inflammageing

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List of abbreviations

AA	Arachidonic acid
AD	Alzheimer's disease
BMI	Body mass index
CLIA	Chemiluminescence immunoassay
CRP	C reactive protein
CVD	Cardiovascular disease
CVM	Cytomegalovirus
DGE	German Nutrition Society (Deutsche Gesellschaft für Ernährung)
DHA	Docosahexaenoic acid
DII	Dietary inflammatory index
DRI	Dietary reference intakes
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid
FA(s)	Fatty acid(s)
FAD	Flavin Adenine Dinucleotide
GLR	Granulocyte/lymphocyte ratio
Hcy	Homocysteine
HoloTC	Holotranscobalamin
ISSFAL	International Society for the Study of Fatty Acids and Lipids
IU	International units
LA	Linoleic acid
LC-MS	Liquid chromatography-mass spectrometry
LDL	Low-Density Lipoprotein
LNA	Linolenic acid
LPS	Lipopolysaccharide
MCI	Mild cognitive impairment
MI	Myocardial infarction
MMA	Methyl malonic acid
MMN	Multi-micronutrient
n	Omega
n.s.	Not significant
NAM	National Academy of Medicine
NFkB	Nuclear factor κ B
O3I	Omega 3 index
PLT	Platelet count

PUFA(s)	Polyunsaturated fatty acid(s)
RBC(s)	Red blood cell(s)
SAM	S-adenosylmethionine
SASP	Senescence-associated secretory phenotype
SCFA(s)	Short chain fatty acid(s)
SD	Standard deviation
SIBO	Small intestinal bacterial overgrowth
SNP	Single-nucleotide polymorphism
SPMs	Specialised pro-resolving mediators
T2D	Type 2 diabetes
T-EMRA	Terminal-end-differentiated cells
TG	Triglyceride
TNF	Tumor Necrosis Factor
U.S.	United States
WBC	White blood cell count
WC	Waist circumference

1. General introduction

The proportion of older people in western industrialised countries is steadily increasing. By 2050, individuals aged 67 and over will make up around 30% of the population in Germany [1]. Science, the prevalence of chronic degenerative and metabolic diseases is higher in older people than in the general population; a demographic shift towards older age is generally accompanied by an increase in the incidence of these diseases [2]. This is because the prevalence of these diseases is higher in older people than in the general population. It also means that already stretched health budgets (currently €138 billion per year in Germany) will have to be stretched further, to €361 billion per year in Germany by 2050 [3]. In conclusion, preventive health care such as nutritional strategies for older people is becoming increasingly important for healthy ageing and for reducing health care costs.

Previous studies have attempted to examine the nutritional status of older people [4–6]. However, there is great heterogeneity in the definition of older people [7]. Ageing is a process that begins with birth and growth and continues through a prolonged period in which physiological changes are largely absent. From a blurred boundary, a process begins in which physiological changes occur and heterogeneity in health status begins to increase. Specifically, the general perception of older people in European countries is defined by 65 years and older [8]. In addition, the American Geriatric Society and the World Health Organisation define a shift from old individuals to oldest old individuals from 80 years and over [9].

Nutrition is known to not only prevent energy and nutrient deficiencies, but also to contribute to long-term health and the prevention of chronic degenerative diseases. Nutrition societies make practical recommendations about food choices and desirable intakes of energy and nutrients, which are not fundamentally different for older people. Irrespective of age, there are large gaps between the recommendations of nutrition societies and the nutritional reality of the German population, especially for the micronutrients vitamin D, folate, cobalamin and n3 FA [10–14]. However, older people are particularly at risk of micronutrient deficiencies due to physical, cognitive and social changes [15].

Current knowledge about the nutritional status of older people is based on studies that have mainly examined the nutritional status of older people living in nursing homes or clinical settings, where the older people studied were already immobile and had severe impairments [16–18]. However, nutritional status is not only determined by dietary intake, but also by several lifestyle factors. In the general population, high levels of physical activity, resulting in low levels of adipose tissue and usually longer duration of UV exposure, which is known to have a beneficial effect on vitamin D, folate and cobalamin status [19–21]. On the other hand, smoking, alcohol abuse, sleep disturbance and social isolation have been shown to have a

negative impact on micronutrient status and quality of life [22, 23]. There is a lack of observations on critical micronutrients such as vitamin D, folic acid, cobalamin and n3 FA in physically active and independently living older people. The question therefore remains whether these older people, who also have a sophisticated understanding of health maintenance, achieve adequate status of typical critical micronutrients.

The next stage is to consider the extent to which healthy and physically active older people may additionally benefit from dietary interventions to prevent nutrient imbalances and maintain health. However, the public health potential of dietary interventions in older people has not been fully explored [24]. Multi-micronutrient (MMN) supplements are a simple and cost-effective dietary intervention strategy for improving nutrient status, and typically cover all essential micronutrients [25]. This is of particular interest given that deficiencies in several micronutrients (e.g. folic acid, cobalamin, pyridoxine, riboflavin) can have adverse effects on metabolic processes, such as increased concentrations of homocysteine (hcy) and methyl malonic acid (MMA), both of which are associated with increased risk of cardiovascular disease (CVD) and mortality in the elderly [26–28]. In addition, there is a progressive decline in the function of the immune system with older age, and a persistent chronic low-grade inflammation occurs [29, 30]. A wide range of micronutrients work together to maintain a well-functioning immune function and reduce inflammation [31]. In contrast, previous studies investigating the potential beneficial effects of dietary interventions on biochemical markers of metabolic health and immune function in older adults have mostly used single micronutrient supplements rather than MMN supplements including physiological doses of n3 FA [31–33]. In addition, the micronutrient status was not assessed using validated long-term status markers. In conclusion, the results of previous observations in older people are still limited and inconsistent, particularly with regard to the modulation of inflammatory markers. It is therefore necessary to evaluate the beneficial effects of MMN supplementation, including n3 FA in an older population using available valid long-term status markers of critical micronutrients.

This science will provide a better understanding of the micronutrient status of the heterogeneous older population and may identify lifestyle and dietary patterns that contribute to healthy ageing. The results will also help to reinforce the beneficial effects of micronutrient supplementation on metabolic health and inflammation.

Aim of this dissertation thesis

This thesis is based on the following research questions, which are addressed in the corresponding scientific publication:

1. **To what extent do healthy, physically active, and independently living older people, who did not consume dietary supplements, achieve adequate status of vitamin D, B_{12} (cobalamin), B_9 (folic acid), and n3 FA?** (*Paper I, chapter 2*)
2. **Does supplementation with a multi-nutrient supplement provide additional benefits for the status of critical micronutrients and metabolic health of older people?** (*Paper II, chapter 3*)
3. **Can multi-micronutrient supplementation positively modulate inflammatory biomarkers in older people?** (*Paper III, chapter 4*)

1.1. Physiological changes and nutrient requirements in old age

Ageing is a physiological process that normally leads to a slow decline in several functions (e.g. gastrointestinal capacity, organ functions, and cognitive performance) that affect nutritional status [34]. In addition, physiological defence and adaptation processes are compromised, which can increase the risk of pathophysiological processes and need of medical drug intake which in turn predict higher nutrient requirements [35].

1.1.1. Body composition

Firstly, the total energy requirement decreases due to physiological changes in body composition, characterised by an increase in adipose tissue and its distribution, which replaces the decreasing muscle mass and hence body water. The loss of muscle mass and function is caused by reduced physical activity and resistance to anabolic exercise (anabolic resistance), decline in anabolic hormones, and a state of chronic low-grade inflammation [36, 37]. As a result of reduced energy requirement less food is consumed, while the micronutrient requirements remains largely the same. Consequently, the nutrient density (defined as the amount of nutrients compared to the amount of energy in a food) of older people's diets needs to be increased, which means a more conscious approach to healthy dietary patterns and well balanced diet.

1.1.2. Anorexia of ageing

Anorexia of ageing is defined as a loss of appetite in older people and describes a decline in the senses of smell and taste, hormonal changes in the regulation of hunger and satiety, reduced salivation and difficulty swallowing [38, 39]. As a result, older people reduce their overall food intake and tend to modify the texture of food to make it more chewy by using intensive and highly processed food preparations, which otherwise leads to a loss of several micronutrients in particular heat volatile vitamins such as vitamin C, folic acid and other B vitamins [40, 41]. Several factors trigger the loss of appetite like social isolation, insomnia or mild depressive disorders, which are all common in the older population [42–45]. As a result, loss of interest in usual activities may further reduce overall dietary intake, outdoor sun exposure and nutrient density of consumed food by favoring convenience or ready-to-eat foods.

1.1.3. Organ function

Several organ systems responsible for the digestion, absorption or synthesis of nutrients experience an age-related decline in function [46]. After ingestion of food, saturation occurs earlier and faster due to a reduced ability of the gastric tissue to stretch. After worth’s chemoreceptors transmit signals to the hypothalamus, resulting in the cessation of food intake.

In addition, gastric emptying after solid and liquid food is slower, due to a decrease in postprandial gastric contractile force, which in turn leads to delayed resumption of food intake and avoidance of snacks [47]. Furthermore, chronic atrophic gastritis is more prevalent in older population and accompanied with a loss of glands in the gastric mucosa (intestinal metaplasia) [48]. Causal for higher prevalence of these chronic inflammatory disease is the higher prevalence in Helicobacter pylori (H.pylori) infection and chronical use of proton pump inhibitors in the old age [49, 50]. This leads to reduced gastric acidity, which affects the absorption of food-bound micronutrients such as cobalamin and folic acid [50]. Kidneys function also undergoes a decline, due to reduced blood flow and loss of glomeruli [41]. This physiological decline can be exacerbated by chronic diseases such as type 2 diabetes (T2D) or CVD [51]. Impaired kidney function can lead to electrolyte imbalances, reduced synthesis of 1.25-hydroxyvitamin D and excretion of metabolic intermediary products, such as hcy [52, 53]. Concerning vitamin D status the endogenous synthesis of 25-(OH)D can be reduced by up to 25% compared with younger adults, as thinner skin and reduced kidney function limit the body's ability to synthesis vitamin D with old age [54].

Age-related changes within the small intestine are concern to the intestinal epithelium which undergoes a rapid turnover. However, tissue renewal and regeneration may be reduced in older age, resulting in a reduced surface area for nutrient adsorption. Reduced physical activity, T2D and hypertension, which are common in older adults, lead to small intestinal bacterial overgrowth (SIBO) [55]. SIBO, which is more common in hospitalized than in healthy older people, is associated with chronic diarrhea, malabsorption and nutritional deficiencies [56]. At least intestinal microbiota changed with age. The composition of the gut microbiota is characterised by changes in the abundance of the two major phyla in humans, Microbiota Firmicutes (Gram-positive bacteria) and Bacteroidetes (Gram-negative bacteria); loss of bacterial diversity and reduced abundance of short chain fatty acid (SCFA) producing bacteria [57]. Finally, changes in the ageing gut microbiota lead to a reduced epithelial barrier to pathogens and are associated with inflammatory bowel diseases.

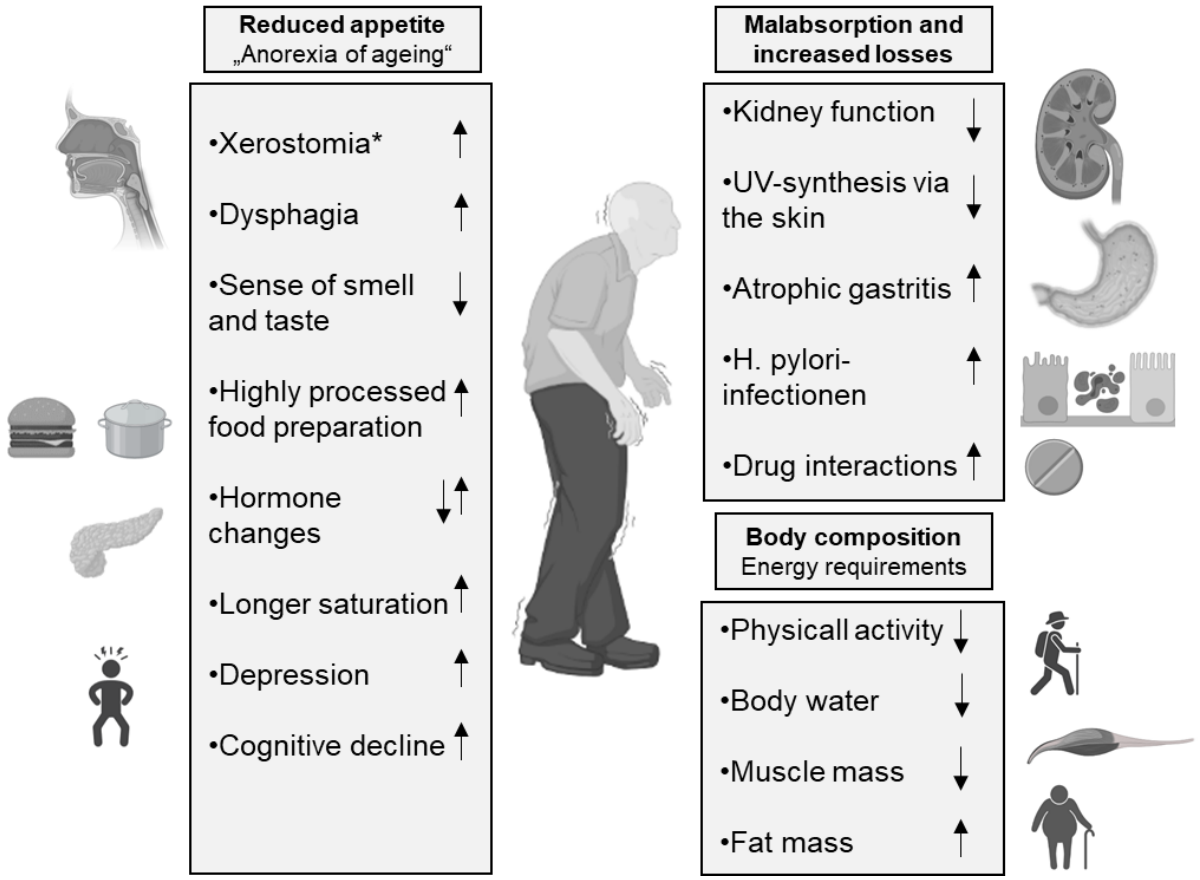

Figure 1: Selection of age-related changes that increase the risk of nutrient deficiencies in older people.
*Xerostomia: dry mouth syndrom

1.1.4. Immune function

The ageing process also affects immune function [29]. This affects both innate and adaptive components of the immune system. To protect the body from infection by pathogens, the innate immune system acts as a first line of defence through natural physical barriers such as the skin and intestinal epithelium. In addition, antimicrobial peptides (e.g. defensins, cathelicidins) and a variety of cells such as neutrophils and macrophages can eliminate pathogens quickly and non-specifically. Secondly the adaptive components of the immune system, which act slower but more pathogen specific, are more able to build up an immunological memory. This consists of certain immune cell differentiations and the production of immunoglobulins that are adapted to a specific pathogen and its control.

As we age, the immune system undergoes structural and functional changes [58]. The age-related decline in immune function is known as **immunosenescence**. However, while the term 'senescence' is primarily used to describe a state of permanent immune cell arrest, the age-related decline in immune function involves multiple mechanisms that are common to several

age-related pathologies, including neurodegenerative and cardiovascular diseases, sarcopenia (defined as loss of muscle mass and function) and cancer [59–61].

In detail, an impaired immune function is firstly characterised by a **reduction in external physical barriers** due to thinner and drier skin and lower amounts of anti-microbial defences as a first line of defence against infection [62]. The natural barriers also include the intestinal mucosa. Leaky epithelial permeability leads to increased pathogen load on the immune system [63]. At the cellular level, immunosenescence affects immune function by reducing the number and functionality of T lymphocytes, which perform a variety of functions in the immune system [64]. Specifically, the involution of thymus tissue which begin after puberty leads to a **reduction in the number of naive T cells**, which are responsible for detecting and eliminating new pathogens [65]. In addition, driven by the cumulative exposure to peristent antigens during life span (e.g. cytomegalovirus [CVM] and Epstein-Barr virus) T cells **loss their ability to differentiate** and to respond adequately to novel pathogens, tumor cells and vaccines [66].

Surface molecules can also be used to assess the degree of differentiation and thus the functionality of cellular immune populations. During ageing, the T-cell population undergoes a remodeling process, resulting in an **imbalanced T-cell population** (e.g. CD4+/CD8+ T-cell ratio), which negatively affects the immune response [67]. In addition, specific T cell populations **lose surface molecules** necessary for costimulation of different types of immune cells. For instance, the costimulatory molecule CD28 and its expression on T cells reflect the aged and weakened immune phenotype of senescent T cells [68]. Human T cells expressing CD28 on their surface are much more responsive to antigens due to a stabilised immune synapse and lower number of antigen engagements required for cell activation [69]. In contrast termed terminal-end-differentiated cells (T-EMRA cells), do not express CD28 on cell surface and accumulate during ageing [70]. Senescent cells also have detrimental metabolic effects, such as increased production of pro-inflammatory mediators, defined as the **senescence-associated secretory phenotype (SASP)** [59]. As a result, in older population elevated levels of pro-inflammatory molecules such as cytokines are found even in the absence of pathogen exposure and have not undergone natural resolution processes. This state of **chronic low-grade inflammation**, also known as **inflammageing**, is associated with a variety of degenerative disease processes and is one of the major hallmarks of accelerated ageing [71, 72]. Cytokines, protein-based signalling molecules, are produced by various cells of the immune system to orchestrate a local or systemic defence response. For the immune response to be adequate, it is essential that the correct amount of immune cells are recruited in response to the recognised pathogen and that the defence response is then actively resolved to minimise damage to the body's own tissues. Therefore, cytokines can have either a pro-inflammatory or anti-inflammatory effect on the immune system, thereby initiating and resolving the inflammatory response. It has been shown that in old age, the signalling effect of cytokines is

unbalanced and no longer adequately to the pathogen and the damage to be detected [73]. This has also been shown, during the COVID-19 pandemic, when an uncontrolled inflammation, the so-called cytokine storm, occurred in the infected older people, leading to acute respiratory distress syndrome and higher mortality in the older population [74]. In contrast, a lower vaccination success can be observed in older people after COVID-19 or influenza vaccination [75]. This means that the immune system response after vaccination is too weak, resulting in insufficient antibody production and a reduced protective effect of the vaccination.

Previous research has focused on the assessment of cytokine concentrations to detect age-related changes in the immune system, as determined by increased levels of pro-inflammatory cytokines (IL-1_b, IL-6, TNF [Tumor Necrosis Factor]$_a$, CRP) and decreased levels of anti-inflammatory cytokines (IL-10, TNF_b) [76]. Another possible method of assessment is to detect imbalances in T-cell subtypes, including measurement of cell surface protein expression [70]. A new approach to detecting changes in immune function in the elderly is to combine cellular and plasmatic biomarkers by applying a cohort-specific score. The INFLA score combines components of both innate and adaptive immune function and may therefore reflect immune function more comprehensively than individual biomarkers, which can lead to inconsistent statements [77].

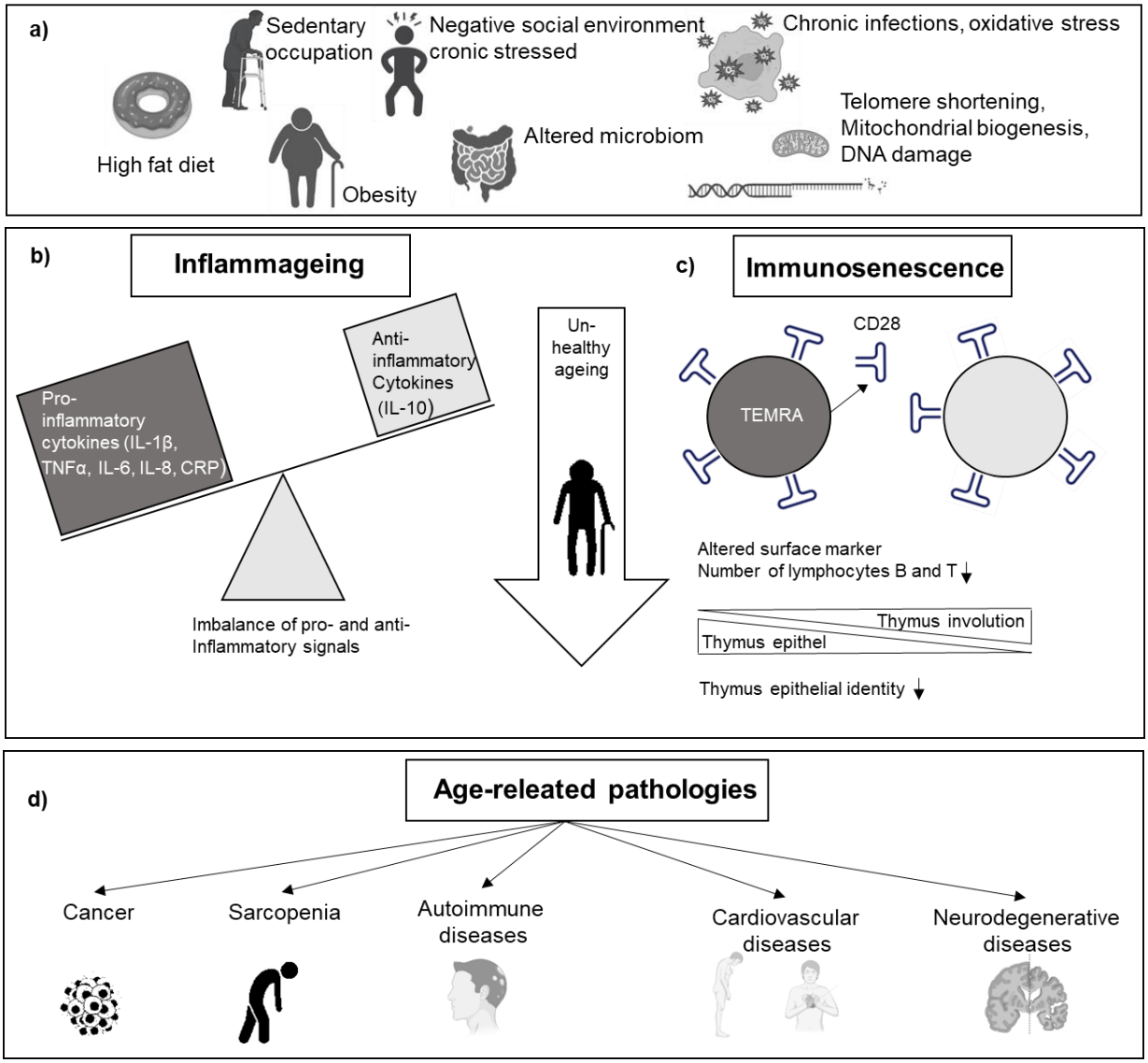

Figure 2: Causes, characteristics and consequences of immune dysfunction in old age. a) Causal factors for unhealthy ageing, b,c) impaired immune function characterised by inflammageing and immunosenescence, d) consequences of unhealthy ageing.

1.2. Critical nutrients in ageing

Nutrient intakes can be assessed by using validated dietary questionnaires to record intakes of individual nutrients and comparing them with recommended intakes [78]. However, dietary assessment depends on the accuracy of the assessment method (e.g. 24-hour recall, 3-day dietary recall, food frequency questionnaires), which is usually retrospective and therefore has some limitations such as subject recall, subjective estimation of portion size and incomplete reporting [79]. Therefore, it is not always possible to find associations between nutrient intake data and nutrient status as assessed by circulating blood biomarkers. In addition, while endogenous pathways contribute to status, for some micronutrients, such as vitamin D, intake cannot match the serum concentration of 25-(OH)D, which is the commonly used status marker for vitamin D [80]. Furthermore, acute inflammation may have a short-term effect on the concentrations of circulating nutrient status marker. Overall, where possible, existing data on valid blood biomarker should be used to identify critical nutrients in the diet of older people. Therefore a critical nutrient is defined as a deficiency in circulating concentrations of a nutrient that may lead to adverse health outcomes [81].

This work explicitly assessed the status of micronutrients, which are also critical in the general population, and whose adsorption or synthesis may be affected by age-associated changes. Finally, valid long-term markers were available for vitamin D status based on serum concentrations of 25-(OH)D, cobalamin status based on holoTC, folic acid status based on RBC folate and the O3I for relative EPA+DHA of total fatty acids in RBCs.

1.2.1. Vitamin D

Endogenous synthesis

The synthesis of 25-(OH)D is based on the steran structure of cholesterol 7-dehydrocholesterol (provitamin D3), which is converted to precholecalciferol (previtamin D3) by UVB radiation in the 290-315 nm range [82]. Previtamin D3 is further metabolised by 25-hydroxylase in the liver to 25-(OH)D. 25-(OH)D is bound to albumin and converted to its metabolic active form 1,25-hydroxyvitamin D in the kidneys [83]. However, due to Germany's geographical location, UVB synthesis of 25-(OH)D by the skin is only possible from March to October, with peak concentrations expected in late summer [84].

Nutrition requirements and recommendations

Dietary sources of vitamin D are ergocalciferol (vitamin D2), which is found in vegetables (such as some mushrooms called shiitake), and cholecalciferol, which is abundant in naturally rich animal foods such as eggs, cod liver oil, fish fat such as salmon, herring, smoked eel and tuna [85]. Dietary sources usually only meet 10% to 20% of the vitamin D requirement and therefore

do not have a significant impact on vitamin D status [10]. Instead the capacity of endogenous synthesis is the main determinant for sufficient vitamin D status.

In the absence of endogenous synthesis, the D-A-CH Society recommends a vitamin D intake of 20 µg per day (1 µg=40 international units [IU]) and is aimed at adults and the people over 65 years of age [86, 87]. It is clearly stated that in case of absense endogenous synthesis this value cannot be achieved through diet and must be achieved by additional vitamin D intake through vitamin D supplementation.

Ensuring adequate serum concentrations of 25-(OH)D is the basis for this recommendation. The National Academy of Medicine (NAM) and European Food Safety Authority (EFSA) consider 25-(OH)D concentrations >50 nmol/L to be sufficient for metabolic health [88, 89]. For the prevention of falls and fractures, some guidelines also recommend 25-(OH)D concentrations >75 nmol/L as desirable, which is particularly important for older people with advanced degenerative bone resorption processes [90].

Dietary intake in the age

In the general German population aged 65 and over, 94% of men and 97% of women did not reach the recommended dietary intake of vitamin D [91]. However, in the general population, vitamin D insufficiency (using serum 25-(OH)D concentration <50 nmol/L) is estimated to range from less than 16% to 80% in Europe, depending on age, latitude, season, sex, smoking status, and the prevalence of obesity and vitamin D supplement use [54].

As already mentioned, older people have a lower capacity for endogenous vitamin D synthesis [92]. It can be assumed that older people, unlike younger people, are not able to maintain sufficient increases in serum 25-(OH)D concentrations during the summer months. However, previous studies have mainly examined older people living in nursing homes or hospitalised older people **(Table 1)**. In addition, the comparison of previous studies is limited by the fact that studies have been conducted in different seasons and in supplemented or unsupplemented cohorts, whereas studies taking seasonal variation into account are rare. As a result, there is a wide range of reported prevalence of vitamin D deficiency (<50 nmol/L), from a low prevalence of 16.1% in summer to 93.9% in winter and among older people in nursing homes (Table 1). However, studies investigating vitamin D status in healthy, independent, physically active older people during the summer are lacking.

Table 1: Previous studies investigating vitamin D status in older people

Reference	Mean 25-(OH)D [nmol/L]	Prevalence of values below the target cut-off of 50 nmol/L [%]	Sample Size (female, male) Mean Age [years]	Status of Supple-mentation	Season
Diekmann et al.[93] 2013, Germany	20.8	93.9	84 F 31 M 86.3	NR	June until December
Klenk et al. [84] 2013, Germany	77.6	16.1	597 F 821 M 75.5	Non-supplemented cohort	August
Shinkov et al. [94] 2015, Bulgaria	17.8	62.5	26 F 40 M 74.5	Non-supplemented cohort	Winter
Conzade et al. [95] 2017, Germany KORA-Age Study	48.3 (all seasons)	60.9 (Febr.-May) 46.9 (Jun-Aug) 45.4 (Sept-Nov)	542 F 537 M 73.9	13 % regular Vitamin D intake	All seasons
Pourhassan et al. [96] 2018, Germany	Summer: 37.8 Winter: 26.3	No suppl.: 85 Using suppl: 35	457 F 222 M 82.1	Subgroup analysation of supplemented older people	All Seasons
Laird et al. [97] 2018, Ireland TILDA Study	Winter: 42.9 Spring: 44.5 Summer: 60.4 Autumn: 52.5 All months: 51.3	Winter: 59.2 Spring: 54.9 Summer: 26.5 Autumn: 41.6 All months: 42.5	2860 F 2496 M 62.9	9.2 % regular Vitamin D intake	All Seasons
Griffin et al. [98] 2020, Ireland	29.7	67.0	176 F 97 M 81.5	NR	Winter

Okan et al. [99] 2020, Turkey	35.8	64.0	13 F 23 M 74.0	Non-supplemented cohort	August
Grootswagers et al. [100] 2020 Dutch	79.9	NR	41 F 40 M 74.2	Non-supplemented cohort	July-August

Abbreviations: NR: not reported, F: female, M: male

Function in health and disease

1,25-dihydroxyvitamin D_3 (calcitriol), known as the metabolically active form of vitamin D, is synthesised in the kidneys. Besides the classical function on calcium and phosphate homeostasis, 1,25-dihydroxyvitamin D_3 have different actions on **musculoskeletal and extraskeletal health**. Possible mechanism of health and disease who are in particularly relevant for older people are listed in **Table 2**:

Table 2: Scientific background of vitamin D in health and disease

Age-related heath issue	Mechanism of action and existing evidence for the benefits of supplementation
Influence of loss of muscle function and muscle mass (Sarcopenia)	**Calcitriol** • Have direct anabolic effect on muscle tissue through improved muscle fibre proliferation and differentiation by binding to a highly specific nuclear receptor in muscle cells and related pathways • Altering intracellular signalling and calcium homeostasis [101] • Low serum concentration 25-(OH)D were seen to increase the risk of sarcopenia [102] • In older adults circulation serum concentration of 25-(OH)D were associated with muscle strength and mass as well as performance [103] • Nevertheless, the relationship between vitamin D status and conditions of sarcopenia should be investigated in healthy community-dwelling older adults [102]
Influence of risk of falls and fractures	**Calcitriol** • Induces the synthesis of osteocalcin, a protein act as an important component of the skeleton, and directly stimulates osteoclasts, leading to bone mineralisation and growth • U-shaped association between serum concentrations of 25-(OH)D and fractures in 1662 community-dwelling men (aged 70-97 years, follow up: 4.3 years), implication that serum concentrations of 25-(OH)D below 36 nmol/L and above 75 nmol/L were associated with the highest risk for fractures [104]

	• Low concentrations of 25-(OH)D were associated with higher risk of fractures studies and reported fractures in existing research • No meaningful effects of vitamin D supplementation in RCTs in community-dwelling older people on BMD, falls and fractures [105]
Influence on cardiovascular health	**Calcitriol** • Have pleiotropic actions on cell proliferation, cell differentiation and cell apoptosis via VDR [106] • VDR is also expressed in vascular tissue, endothelial cells and cardiomyocytes [107] • Has been shown to remodulate cardiac muscle fibres and attenuate angiogenesis and macrophages to foam cell formation [108] • The vast majority of large epidemiological studies (Lyric Study [106], Womens health study [109], VIDA study [110], VITAL study [111], FIND study [112], D Health Trial [113]) examining the effect of vitamin D supplementation on cardiovascular endpoints in older population (age ranged: 50-84 years) have found no convincing evidence of a preventive effect
Influence on immune function	**Innate immune function [31]:** • Calcitriol work in concert with several micronutrients to maintain a well-functioning immune system • For instance with vitamin A to support the epithelial barrier function of the gut • Anti-inflammatory effect of vitamin D is supported by the discovery of the vitamin D activating enzyme 1-alpha-hydroxylase (CYP27B) and the expression of the vitamin D receptor on various immune cells • Stimulate the synthesis of defensins and cathelicidins in immune cells, which have direct antimicrobial activity and can also act as metabolites for the recruitment of immune cells to the site of infection **Adaptive immune function [31]:** • Calcitriol mainly act inhibiting on immunce cell differentiation and proliferation but also maintain normal functioning by increasing oxidative burst potential against pathogens • Differential promotion and inhibition of T cell subsets (inhibition of TH1 cell activity, increased production of Treg), as well as the increased expression of anti-inflammatory cytokines (IL-10 TGFβ,), vitamin D is known to predominantly slow down an activated immune system • Increased antibody production after influenza vaccination under the condition of vitamin D supplementation **Inflammation** • Cross sectional: significant correlation between serum 25-(OH)D concentrations and CRP concentrations in subjects with and without inflammatory diseases [114] • RCT: vitamin D supplementation in an older cohort have already been shown in reduced levels of circulating pro-inflammatory cytokines [115] • Individual RCTs also showed significant effects on pro-inflammatory cytokines after vitamin D supplementation in subjects with T2D

	• Systematic review: effects of vitamin D supplementation on inflammatory cytokines (CRP, TNF_a, IL-6) in 81 trials involving 9,276 participants. 79% of these trials showed no significant effect on any of these inflammatory markers [116]

1.2.2. Folate and cobalamin:

Unlike vitamin D, dietary intake of folic acid and cobalamin and conditions that affect the absorption mechanism play an important role in the development of critical folate and cobalamin status in the older population, so it's important to describe the physiological absorption process in more detail.

Metabolism

Folic acid reflect a family of many molecular species [117]. The active form is the fourfold reduced tetrahydrofolic acid. Following protein-bound release of folic acid in the stomach, folic acid is absorbed in the intestine in symport (proton-coupled folate transporter) with protons or by ion exchange (reduced folate carrier). In plasma, 5-methyltetrahydrofolic acid circulates free or bound to folate binding protein. Folic acid accumulated in target cells for example in red blood cells by polyglutamation [118]. Because of the longevity of erythrocytes, RBC content is also a valid measure of folic acid intake over the past 120 days [119]. Cobalamine also reflect several molecular species differ in binded ligands. **Methyl cobalamin** is the metabolic active form whereas cynaobobalamin often placed in dietary supplements [83]. Cobalamin in foods general bound on proteins [83]. The protein bond is cleaved in the acidic milieu in the stomach. Age-associated diseases such as atrophic gastritis can lead to an elevation of gastric pH and reduced protein cleavage from dietary cobalamin. In addition, cobalamin can also be present in food as a free molecule, in which case it is initially bound to haptocorin, which is already present in the saliva in the mouth, or it binds to cobalamin in the stomach after the protein has been cleaved. As the next step in the complex absorption mechanism of cobalamin, the intrinsic factor (IF), produced in the parietal cells of the gastric mucosa, binds to cobalamin released by pancreatic enzymes in the anterior (duodenum) and middle (jejunum) parts of the small intestine at the latest [120]. Binding is essential and another critical point for the subsequent terminal absorption of cobalamin in the terminal ileum. Afterwards, cobalamin is mainly bound to transcobalamin II resulting in the circulating transport form of holo-TC, which is also a valid biomarker for assessing long-term cobalamin intake [121, 122].

Nutrition requirements, recommendations

The recommended dietary intake for folic acid equivalents is set at 400 µg per day and for cobalamin at 4.0 µg per day, and both recommendations did not differ between the general population and the elderly [87]. To meet the recommended intake of folic acid, unprocessed

green leafy vegetables, whole grains and legumes are suitable due to their high levels of folic acid [85].

Dietary intake in the age

In the German general population, 89% of men aged 65 years and over and 91% of women aged 65-80 years did not meet the recommended intake of folic acid [91, 123]. Among institutionalised older people, the proportion of people not meeting the recommended intake was higher at 97% for women and 99% for men [124]. The main sources of folic acid in the German national survey were folic acid from non-alcoholic drinks, followed by cereals and green vegetables, although milk and dairy products and fruits also contributed to achieving 400 µg of folic acid per day.

In Germany, 9.8% of men and 26.3% of women aged 65-80 years did not meet the recommended intake of cobalamin [91, 123]. Among institutionalised older people, the proportion was clearly higher at 48% for both sexes [124]. The main sources of this water-soluble vitamin are meat, fish, eggs and dairy products [85].

Previous observations assessing the prevalence of folic acid and cobalamin deficiency differ by a diversity of available biomaker (Listed in **Table 3**) and the used cut-offs.

Table 3: Overview of biomarker for assessment of folate and cobalamin status

Biomarker	Characteristic	Normal range
Serum concentration of folic acid	• Widely used in clinical settings • Shortly influenced by diet [125] • Does not reflect bioavailability of cellular folic acid	• Normal serum concentration is above 6.8-22.7 nmol/L (3-10 ng/mL) [126, 127]
RBC folate	• Reflect long time intake over past 120 days [112] • First line biomarker for the assessment of folate status [128]	• Normal concentration in RBC depending on assay used [127, 129]
Serum concentration of cobalamin	• Widely used in clinical settings • Does not reflect bioavailability for cellular uptake and cellular cobalamin status [121]	• Assays ranged from <150 pmol/L and <200 pmol/L, classified as low [130, 131]
HoloTC	• First line marker for biologically active fraction of cobalamin, in particular for older people [121]	• Normal serum concentration is above 35 and 50 pmol/L [132, 133]

	• Unaffected by estrogen variations [121]	
Hcy	• Metabolic marker for folate and cobalamin status [134] • Plasma concentrations should be used to determine (serum concentration also contain hcy bound on cell components [121] • Decreased sensitivity may be expected with impaired renal function, thyroid dysfunction or pyridoxine deficiency [121]	• Normal plasma concentrations between 5-15 µmol/L [135]
MMA	• Metabolic marker for cobalamin [136] • Decreased sensitivity may be expected with decreasing renal function [137]	• Serum concentrations: 260-350 nmol/L depending on assay selected [135]
4cB12* [138]	• Aggregated marker of cobalamin status included metabolic and functional markers • Corrected for age and folate status	• Increased cobalamin status: 4cB12: >1:5 • Adequate: 4cB12 -0.5-1.5 • Possible deficiency: 4cB12: -1.51- (-2.5) • Probable deficiency: 4cB12: < (-2.5)

*: $4cB12 = \log 10\left(\frac{HoloTC \times B12}{MMA \times tHcy}\right) - \left(\frac{3.79}{1 + \left(\frac{age}{230}\right)^{2.6}}\right)$

The prevalence of folic acid deficiency in older people, as measured by serum concentrations of folic acid or RBC folate, ranged from almost none to about half of the study population (**Table 4**). Prevalence of cobalamin deficiency in older people ranged between 4.2% and 88% assed by serum concentration of cobalamin, holo-TC and combinated marker such as 4cB12.

Table 4: Previous studies investigating cobalamin and folate status in older people living in different settings

Reference	Prevalence of values below the used cut-off [cut-off]	Sample Size (female, male) Mean Age [years]	Status of Supple-mentation	Living situation

Wolters et al. [139] 2003, Germany	Serum cobalamin [<258 pmol/L]: 42.9% Serum folate [<7 nmol/L]: 0% RBC folate [<320 nmol/L]: 2.3% MMA [>271 nmol/L]: 9.6% Hcy [>12 µmol/L]: 17.4%	178 F 0 M 63.2	Non-supplemented cohort	Community-dwelling individuals
Gonzales-Gross et al. [140] 2007, Spain	Serum cobalamin [<150 pmol/L]: 15.8% Holo-TC [<45 pmol/L]: 39% Serum folate [<13.6 nmol/L]: 48.9% RBC folate [<400 nmol/L]: 18.1% MMA [>500 nmol/L]: 32% Hcy [≥15 µmol/L]: 54.1%	136 F 82 M 78.5	NR	Nursing home
Hermann et al. [141] 2005, Germany	Holo-TC [<35 pmol/L]: 28% Serum folate: mean: 13.6 nmol/L prevalence of deficiency not reported	F+M 228 81	NR	Community-dwelling and institution
Yildirim et al. [142] 2015, Turkey	Serum cobalamin [<150 pmol/L]: 64% Serum folate [<6 nmol/L]: 10.9%	413 F 414 M 70.9	excluded	Community dwelling
Miles et al. [143] 2016, UK	Serum cobalamin [<300 pmol/L]: 88% Holo-TC: mean 49.3 pmol/L prevalence of deficiency not reported Serum folate: mean: 17.6 nmol/L prevalence of deficiency not reported	107 F 94 M 80.0	Non-supplemented cohort	Community-dwelling
Sahin et al. [144] 2016, Turkey	Serum cobalamin [<200 pmol/L]: 6.1% Serum folate [<4 nmol/L]: 7.1%	159 F 98 M 78.5	NR	Nursing home
Conzade et al. [32] 2017, Germany	Serum cobalamin [<221 pmol/L]: 27.3% Serum folate [<13.6 nmol/]: 8.7%	542 F 537 M 65-93	folic acid: 10.8% serum cobalamin: 10.5% of the cohort	572 non frailty 377 pre-frailty 46 frailty older people

Xu et al. [145] 2020, China	F: 60-80 years: Hcy [≥15 µmol/L]: 16.3% F: >80 years: Hcy [≥15 µmol/L]: 30.7% M: 60-80 years: Hcy [≥15 µmol/L]: 45.8% M: >80 years: Hcy [≥15 µmol/L]: 70.2%	4.692 F 3.180 M 60-80	NR	Nursing home
Zhu et al. [146] 2020, Netherland	Serum cobalamin [<150 pmol/L]: 4.2% Serum folate [10.2 nmol/L]: 19.5%	803 F 802 M 65	15% overall	NR
Lavrisa et al. [147] 2021, Slovenia	Serum cobalamin [<150 pmol/L]: 10.4% Serum folate [<7nmol/L]: 18.5% Hcy [≥15 µmol/L]: 39.9%	203 F 213 M 68.7	27.6% taking MMN supplements	Community-dwelling
Porter et al. [49] 2021, Ireland	Healthy: 4cB12 calculated using serum total vitamin B-12, serum holoTC, plasma homocysteine, serum folate, and age to provide a combined indicator value: 15% PP users: 4cB12: 25% AD: 38 %	2219 F 1.080 M 72.8	Non-supplemented cohort	NR

Abbreviations: NR: not reported, F: female, M: male

Function in health and disease

The metabolic functions of folate and cobalamin in health and disease are closely linked. Both vitamins are involved in the transfer of methyl groups during the metabolism of amino acids and nucleic acids [81].

Micronutrient deficiencies in folate, cobalamin, pyridoxine and riboflavin lead to the accumulation of circulating hcy and MMA [148]. Hcy is an amino acid that is synthesised as an intermediate product of methionine metabolism. In the case of low methionine intake and for regeneration of **tetrahydrofolic acid**, hcy is remethylated to methionine. The conversion requires **folic acid** (in the form of 5-methyltetrahydrofol **[5-MTHF]**) as a methyl group substrate and **cobalamin** as an enzymatic cofactor for **methionine synthase**, which remethylate the methyl group from 5-MTHF to hcy, resulting in methionine [148].

That's why cobalamin deficiency leads to accumulation of 5-MTHF and depletion of tetrahydrofolic acid, which is also essential for DNA synthesis and cell replication.

Despite, the remethylation to methionine hcy can be metabolised to cysteine and ultimately excreted via the urine (transulfuration pathway) [149]. This catalysis requires **pyridoxine** as an enzymatic cofactor of the **cysthathionine-beta-synthase**. **Riboflavin** in the form of flavin adenine dinucleotide (FAD) also play a role in hcy metabolisms and acts as a cofactor for methylenetetrahydrofolate reductase (**MTHFR**). MTHFR regenerates 5-MTHF, which is then available for the metabolism of hcy. In particular, in the case of a polymorphism and a reduced enzyme activity of MTHFR, the riboflavin status can play an important role in the hcy metabolism [150].

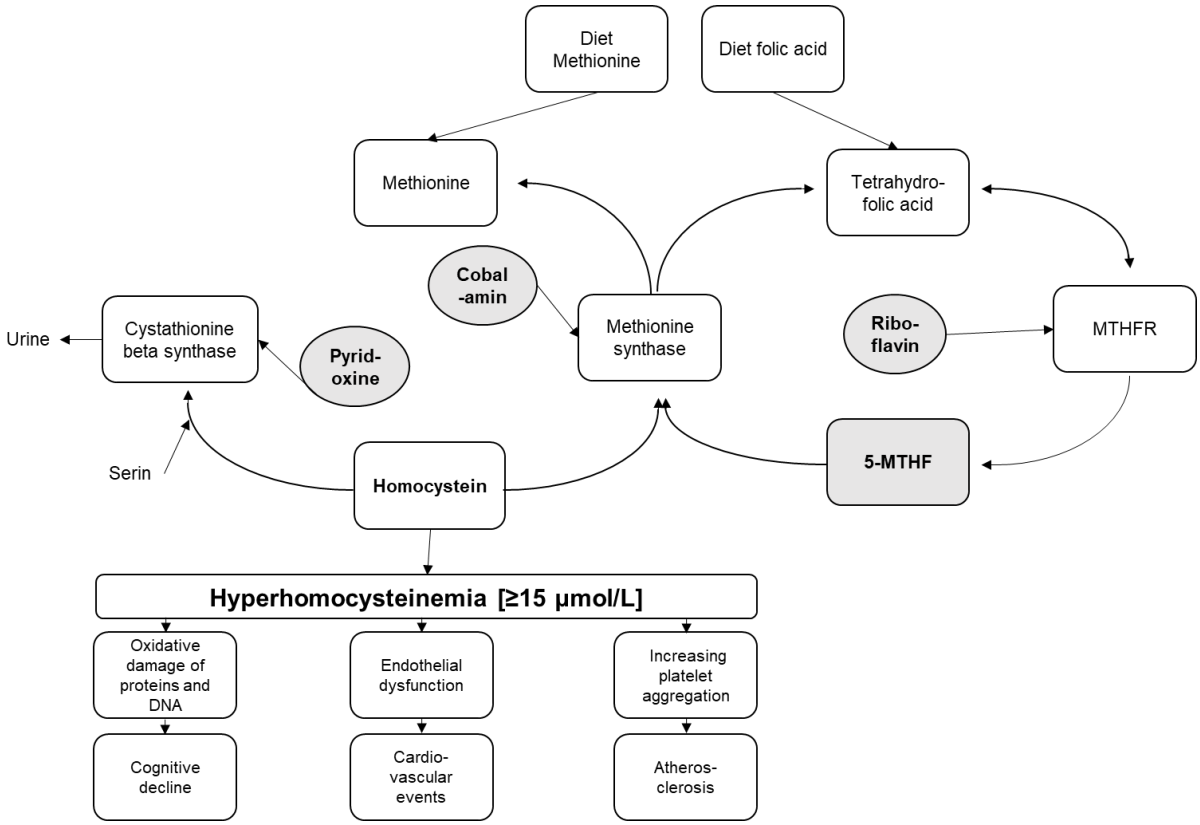

Figure 3: Interplay of B vitamins within the homocysteine-methionine cycle.

In addition, cobalamin has a second function within mitochondria as a co-factor of the methylmalonyl CoA mutase. The enzyme is an important component in the metabolism of amino acids and fatty acids for the production of energy from these substrates. In this process, it provides essential molecular conversion for entry into subsequent metabolic pathways. Enzymatic inactivity caused by cobalamin deficiencies results in the accumulation of MMA [135].

Hyperhomocysteinemia

An increase in circulating hcy concentrations ≥15 µmol/L is defined as hyperhomocysteinemia (hhcy). Blood concentrations ≥15 µmol/L being a commonly used cut-off to define hhcy in older people, although some authors have reported hhcy using lower cut-offs (>12 µmol/L) [133,

151]. The prevalence of hhcy in older people ranged between 17.4% and 54.1% depending on used cut-off and clinical setting (**Table 5**).

Table 5: Health consequences of hyperhomocysteinemia

Age-related heath issue	Mechanism of action
Effects of elevated homocysteine concentrations on CVD	• Hcy react with low-density lipoprotein (LDL) particles and macrophages, which contribute to the metabolisation of oxidised LDL and formation of foam cells, which in turn secrete reactive oxygen metabolites [152] • Hcy increased oxidative stress causes endothelial and mitochondrial dysfunction, telomere shortening and trigger inflammation in smooth muscle cells [153] • Hcy have direct toxic interaction with endothelial cells, leading to apoptosis [154] • Hcy have direct prothrombotic effect, inhibiting protein C, which in turn leads to increased fibrin formation [155] • It is still controversial whether hhcy dependent or independent of folate and cobalamin status is a causal factor of the development of CVD [156, 157] • In a retrospective review of the NHANES III (National Health and Nutrition Examination Survey III) and MESA (Multi-Ethnic Study of Atherosclerosis) datasets, hcy concentrations above 15 µmol/l significantly predicted cardiovascular events, especially in patients at intermediate risk for CVD [158] • It can be assumed that hhcy exacerbate the negative effects of risk factors of CVD, such as smoking, hypertension, and lipid and lipoprotein metabolism
Effects of elevated homocysteine concentrations on neurodegenerative disorders	• The neurotoxicity of hcy is based on increased oxidative stress, DNA damage, thiol group transfer or direct interaction with proteins leading to cytotoxicity and apoptosis [154] • In case control study hcy concentration > 14 µmol/L double the risk of Alzheimer's disease (AD) in older people with dementia [159] • Methionine to hcy status was associated with dementia development and structural brain changes in longitudinal observation over 6 year of follow up [160] • Cross sectional assessment hcy concentration > 15 µmol/L was significantly associated with mild cognitive impairment (MCI) and AD, whereas no association existed between low serum concentrations of vitamin B_{12} and AD or MCI [159]

Table 6: Scientific background of folate and cobalamin in health and disease

Age-related heath issue	Mechanism of action and existing evidence for the benefits of supplementation
Influence of B vitamins on hhcy	• Supplementation of B vitamins (pyridoxine, folic acid, and cobalamin) is effectively to reduce elevated hcy concentrations [156] • Supplementation of folic acid appears to have the strongest effect on elevated hcy concentrations [161] • Additional intake of cobalamin induces further reduction, particularly in the case of serve cobalamin deficiency [161] • Riboflavin may have additional benefits in case of genetic polymorphisms of MTHFR [162] • No evidence that the associated reduction in hcy with B-vitamin supplementation has a favorable effect on CVD risk
Influence of B vitamins on inflammation	• Due to the key role of both vitamins in methylation processes, folic acid and cobalamin are involved in the maintenance of innate and adaptive cell populations [31] • In particular, in response to pathogen exposure, folic acid and cobalamin are necessary for high proliferation and differentiation of various immune cell populations [163] • Deficiency of one or both vitamins can lead to immune cell imbalances and reduced antibody production following vaccination [164] • Cobalamin increases the activity of cytotoxic immune cells [165] • Hhcy can trigger inflammatory processes along with increased concentrations of pro-inflammatory cytokines [166]
Influence of B vitamins on macrocytic anemia	• Folic acid and cobalamin are essential for the metabolism of hcy to methionine, which is a precursor of S-adenosylmethionine (SAM), a key methyl donor molecule, among other methylation processes necessary for DNA synthesis and erythropoiesis [167] • Nutritional deficiencies account for a third of all anemias in older population in the USA, noticed that half of which are secondary to iron deficiency [168, 169] • Anemia (defined as: hemoglobin: men <13 g/dL, women <12 g/dL) is common in the older population ranging from 6.1 to 55.7% [170] • Inconsistent cross-sectional observations between low cobalamin or folate status and prevalence of anemia in older people [171] • Well-designed large trials investigating the effect of cobalamin and folic acid supplementation in older people on haematological parameters are scarce, and no beneficial effects can be concluded [172]

1.2.3. Omega-3 fatty acids:

Metabolism and endogenous synthesis

Omega-3 fatty acids (n3 PUFA) are long-chain, polyunsaturated fatty acids with more than 20 carbon atoms and at least three (up to six) double bonds, which cannot be synthesised de novo by humans and must therefore be obtained from the diet [173]. Alpha-linolenic acid ($_{a}$LNA, 18:3n3), eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3) are the most discussed FA in health and disease in older people due to their multiple mechanisms of action [174]. There is a metabolic pathway to convert EPA and DHA from dietary $_{a}$LNA, which take place in the endoplasmic reticulum and peroxisome. However, the necessary elongation steps are in competition with the same enzymes in the conversion of n6 FA (linoleic acid [LA, 18:2n6] converted to arachidonic acid [AA, 20:4n6]). In conclusion, dietary LA intake, the precursor of n6 FA elongation is a competitive inhibitor for the convertion of dietary $_{a}$LNA to EPA and DHA. In addition, DHA can be converted to a small and irreversible extent to EPA. Total elongation steps for conversion within the n3 PUFAs and n6 PUFAs are generally low efficiency [175].

Nutrition requirements, recommendations and dietary intake situation

Recommended daily intakes of EPA and DHA for older people are similar to those for the general adult population, ranging from 140 mg/day (NAM) to 500 mg/day (International Society for the Study of Fatty Acids and Lipids, ISSFAL), depending on the nutritional society [176, 177]. The average intake of EPA and DHA in Germany is low, at 65-78 mg for EPA and 107-135 mg for DHA, which is about 2.5 to 5 times lower than the recommended intake of 500 mg EPA+DHA per day [178]. However, there is no age-specific assessment of EPA and DHA intake in Germany. The main sources of n3 PUFA intake are of marine origin (e.g. cold-water fish such as salmon, mackerel, herring or tuna) [179]. Within the German population, average fish consumption was slightly higher in men over 65 years than in younger men, but the study did not investigate the type of fish consumed and did not provide information on the n3 PUFA content [91]. In addition the bioavailability of consumed n3 PUFA can be reduced by matrix effects (presence or absence of other food ingredients e.g. overall fat intake or Calcium content), health status or possibly by the age [180, 181].

Status assessment in older population

An established marker of n3 PUFA intake is the O3I, which reflects the relative EPA+DHA content of total fatty acids in red blood cells [182]. The oral administration of EPA and DHA can alter the contents of AA in biological membranes or vice versa [183]. Previous observations suggested that the incorporation of DHA into plasma lipids was improved in healthy older people compared with younger people [184]. In addition, a higher conversion rate of DHA to

EPA was observed in very old compared to young adults [185]. It can be speculate that the concentrations of EPA and DHA in phospholipids increase with age [186].

However, there is a need for additional evidence using established biomarkers of n3 PUFA intake. Data from several studies suggests that a desirable target for O3I is >8%, and levels ≤4% are described as undesirable for cardiovascular health [173, 182]. In 2016, the O3I of 54 different countries worldwide was examined and it was pointed out that the intake of EPA and DHA is insufficient in many parts of the world, including Germany were the O3I ranged between 3.4% and 5.4% [187]. However, studies investigating O3I in older people living in different settings are still lacking. In addition, it is unclear whether healthy, unsupplemented older people can benefit from supplementation with physiological doses of n3 FA in terms of increasing O3I to the desirable 8%

Function in health and disease

Previous research has shown that the n3 PUFAs EPA and DHA have overlapping effects and play an important role in altering blood lipid profiles and membrane lipid composition, hydroxylation into bioactive metabolite known as specialised pro-resolving mediators (SPMs) as well as influencing eicosanoid biosynthesis, which in turn modulates inflammatory processes, cell signalling cascades and gene expression, thereby positively influencing health [188, 189].

Table 7: Scientific background of n3 FA in health and disease

Age-related heath issue	Mechanism of action and existing evidence for the benefits of supplementation
Influence of blood lipids and CVD	• Low O3I (O3I<4) was associated with increased risk and desirable O3I (O3I >8%) was associated with lower risk of CVD [182] • Dietary or supplemental n3 FA is beneficial in reducing CVD-associated risk factors, such as elevated triglyceride (TG) concentrations, in a dose-dependent manner [190, 191] • RCTs evaluating the beneficial effects of n3 FA supplementation on the risk of CVD found predominantly protective effects, whereas meta-analyses found almost no protective effect [192–195] • Higher doses of n3 FA (2-4g per day) appear to be safe for reducing CVD events in several settings [196]
Influence of muscle function and muscle mass (sacropenia)	• n3 FA may have indirect anabolic effect through cell membranes composition changes which in turn influence endocytosis and exocytosis from nutrients and neurotransmitter [197] • n3 FA increase activation of mTOR anabolic signaling pathway via improving insulin sensitivity [198]

	• n3 FA reduce inflammation, which is an independent causal for sacropenia [183, 199]
Influence of cognitive function	• Brain DHA concentrations decrease with age, especially among older people with AD [200] • Reduced dietary intake of n3 FA, low brain levels of DHA, DHA in plasma phosphatidylcholine, ratio of n3 to n6 FA in RBC were all associated with accelerated cognitive decline in older cohorts [201]
Influence of inflammation	• Higher amount of n3 FA compared to n6 FA make cell membranes more fluid, which in turn modulates protein function and pro-inflammatory signalling events [202, 203] • Free EPA and DHA can inhibit inflammatory processes by converting via multiple hydroxylation's into bioactive metabolites such as resolvins, protectins and maresins, collectively known as SPMs [189] • Free EPA and DHA inhibit leukocyte chemotaxis, adhesion molecule expression and leukocyte-endothelial adhesive interactions [183] • Cross sectional studies already show negative associations of O3I and several type of inflammatory biomarkers (CRP, TNF $_{a}$, IL-6, neutrophil to lymphocyte ratio (NLR ratio) [204, 205] • EPA and DHA decreased LPS (lipopolysaccharide)-stimulated production of IL-6,IL-8 and TNF_a by cultured human endothelial cells [206] • n3 FA supplementation via fish oil is beneficial to increase the concentration of the anti-inflammatory cytokine IL-10 [207] • Most human RCTs showed inconsistent effects of different pro-inflammatory cytokines or no effect at all [174, 208–210] • In contrast, some studies demonstrated anti-inflammatory properties by significantly reducing CRP or cytokine levels in middle high or high doses of n3 FA [211, 212] • It is generally thought that supplementation with EPA+DHA at physiological doses may be anti-inflammatory. However, the evidence is inconsistent based on different markers defined for low-grade inflammation [30]

2. Paper I

Folate, vitamin B_{12} and vitamin D status in healthy and active home-dwelling people over 70 years

Authors: Felix Kerlikowsky; Jan Philipp Schuchardt; Andreas Hahn

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Folate, vitamin B12 and vitamin D status in healthy and active home-dwelling people over 70 years

Felix Kerlikowsky[1], Jan Philipp Schuchardt[1] and Andreas Hahn[1*]

Abstract

Background Ageing is characterised by physiological changes that can affect the nutrient availability and requirements. In particular, the status of vitamin D, cobalamin and folate has often been found to be critical in older people living in residential care. However, there is a lack of studies investigating the status of these nutrients in healthy and active home-dwelling elderly people.

Methods The aim of this cross-sectional study was to assess the status of vitamin D based on serum concentrations of 25-hydroxycholecalciferol [25-(OH)D], cobalamin based on serum concentrations of holotranscobalamin (holoTC) and folate based on red blood cell (RBC) folate in unsupplemented, healthy and active German home-dwelling subjects ≥ 70 years of age (n = 134, mean ± SD: 75.8 ± 4.5 years). Dietary intake was assessed by 3-day food recalls. The study was conducted between March and November of 2021 (during the COVID-19 pandemic).

Results The mean 25-(OH)D concentration was high at 85.1 ± 26.0 nmol/L, while the majority of women (92%) and men (94%) had 25-(OH)D concentrations ≥ 50 nmol/L. Less than 10% of men and women had 25-(OH)D concentrations < 50 nmol/L. The mean holoTC concentration was 88.9 ± 33.7 pmol/L (94.8 ± 34.6 pmol/L in women and 73.6 ± 25.6 in men). Only 8% of the women were cobalamin deficient (< 50 pmol/L holoTC) compared to 22% of the men. The mean RBC folate concentration was 831 ± 244 nmol/L, while the prevalence of folate deficiency was 10%. Linear regression analysis showed that only folate equivalent intake was associated with the relevant nutrient status marker.

Conclusion Our findings suggest that healthy, independently living older people with high levels of education, physical activity, and health awareness are not necessarily at higher risk of vitamin D, folate and cobalamin deficiency. Further studies are needed to verify these findings and to identify lifestyle and dietary patterns that can predict adequate nutrient status for healthy ageing.

Trial registration This study is officially recorded in the German Clinical Trials Register (DRKS00021302).

Keywords Nutrient status, HoloTC, RBC Folate, Ageing

*Correspondence:
Andreas Hahn
hahn@nutrition.uni-hannover.de
[1]Institute of Food Science and Human Nutrition, Leibniz University Hannover, Hannover, Germany

Introduction

The supply of nutrients such as vitamin D, cobalamin and folate is sometimes critical for the general population in many countries around the world [1]. Older people in particular are at risk of insufficient intake or deficiency of these nutrients due to age-related dysfunction (e.g., reduced mucosal integrity) or an unbalanced diet [2]. This is supported by the fact that diet-related metabolic disorders, such as type 2 diabetes or cognitive and neuromuscular dysfunction, increase with age [1, 3].

1,25-dihydroxyvitamin D, the active form of vitamin D, is important not only for bone and tooth formation, but also for the immune system and the neuropsychiatric function [4]. The primary determinant of vitamin D status is not dietary intake, but endogenous synthesis, which may be insufficient due to reduced sun exposure during the winter months and decreased capacity with age. In conclusion, the minimum serum 25-(OH)D level can be observed in February and March, and the maximum in late summer [5].

There is an ongoing debate about the target concentrations of circulating 25-(OH)D needed to maintain health, and suggested cut-off values in scientific publications and advisory bodies vary [6]. The National Academy of Medicine (NAM) and European Food Safety Authority (EFSA) consider 25-(OH)D concentrations>50 nmol/L to be sufficient for metabolic health [7, 8]. For the prevention of falls and fractures, some guidelines also recommend 25-(OH)D concentrations>75 nmol/L as desirable, which is particularly important for older people with advanced degenerative bone resorption processes [9, 10].

The supply of cobalamin may be critical for the elderly due to its complex absorption process, which may be affected by age-related disorders [11–13]. Dietary intake may be sufficient for older omnivores, but reduced absorption due to atrophic gastritis as well as *Helicobacter pylori* infection, chronic use of proton pump inhibitors or lack of intrinsic factor may lead to deficits in cobalamin status [14]. Because clinical symptoms such as macrocytic anaemia and neuropathy are not immediately apparent, cobalamin deficiency often goes unrecognised for a long time [12]. It is therefore important to screen also apparently healthy elderly individuals for cobalamin deficiency using valid long-term markers such as holotranscobalamin (holoTC) [15].

The main reason for folate deficiency is an unbalanced diet that is low in unprocessed vegetables, whole grains, and legumes, or vitamin losses during meal preparation. In the general population the dietary intake of folate (equivalents) is often below the recommendations [16, 17], which can lead to inadequate folate status. In addition, age-related changes lead to a decreased sense of hunger, while satiety signals become faster and stronger [2]. In conclusion, despite a good health state, the frequency of meals and the total amount of food consumed may decrease in older people. In addition, older people avoid eating raw and unprocessed fruits and vegetables. Instead, intense heating and gentle cooking make it easier to consume fruits and vegetables, but reduce the bioavailability of folate from foods. Folate deficiency can lead to elevated homocysteine levels, which are associated with a higher risk of cardiovascular disease and cognitive decline in older people [18, 19].

There is a lack of data on the vitamin D, cobalamin, and folate status in older but otherwise healthy older people, especially those aged 70 years and older living independently. The associations between education, physical activity, health attitudes and awareness, alcohol consumption, smoking, and medications on the one hand and the vitamin D, cobalamin, and folate status on the other hand in the elderly population are rarely investigated. As a result, it is difficult to draw conclusions about the nutritional status of at-risk groups such as the elderly. In addition, nutritional randomised controlled trials (RCT) in older people tend to reach those who already have a sophisticated understanding of health maintenance and disease prevention, making it difficult to draw conclusions about the population as a whole.

Therefore, the primary aim of our study was to evaluate the vitamin D, cobalamin, and folate status in unsupplemented, healthy, independently living elderly people≥70 years of age using reliable, state-of-the-art status markers. The secondary aim of the study was to investigate associations of the vitamin status markers with the age and intake of specific dietary food groups.

Materials and methods

Study design and participants

The cross-sectional evaluation was performed using baseline data from a larger study. The original study was a randomized, double-blind, placebo-controlled trial involving 134 subjects aged≥70 years with the overall aim of assessing and improving the status of critical nutrients in older people. Sample size was calculated using an expected drop-out rate of 10%, a significance level of 5%, and a power of 80%. To detect differences in the two-sided t-test between the verum and placebo groups with a Cohen's effect size of 0.5, a case number of 60 subjects per group (n=120 in total) was obtained. Details of this study have been reported elsewhere [20].

Briefly, the baseline data collection was conducted in accordance with the guidelines of the Declaration of Helsinki and carried out at the Institute of Food Science and Human Nutrition in Hannover, Germany (hereinafter referred to as the "Institute") between March 2021 and November 2021. The ethic committee of the medical chamber of Lower Saxony (Hannover, Germany)

approved all procedures. Written informed consent was obtained from all participants prior to their enrolment.

Participants were recruited by announcing the study in the local press, at senior networking centres and volunteer clubs. All interested participants were screened for their health status and controlled for inclusion and exclusion criteria, 134 subjects were invited to the Institute for examination by Institute's staff members (Fig. 1). The main inclusion criteria were an age ≥ 70 years and an independently, home-dwelling living situation. Exclusion criteria were defined as current cardiovascular, metabolic or malignant disease as well as current or up to three months past use of dietary supplements. All interested subjects were asked by telephone about their use of dietary supplements before being invited to the study. If they did not use any supplements, they were invited to the study day, where they were asked a second time about their use of supplements in the previous three months. In case of a conflict of interest, interested subjects were excluded from the study.

Food and nutrient intake

Participants completed 3-day food recalls, including two consecutive weekdays and one weekend day. The PRODI6.4® dietary software based on the German Federal Food Code 3.02 (Nutri-Science GmbH, Freiburg, Germany) was used to analyse the amount of food, food groups and nutrient-specific data such as energy, macronutrients, minerals and vitamins in the reported diet over three days. The 3-day food recall information was also used to assess the consumption of fortified and/or energy-reduced foods. The dietary questionnaires were scored by trained nutritionists of the Institute. Food and food group intakes were reported and calculated based on energy adjustment using the residuum method previously described by Willet et al. [21].

Lifestyle and health behaviour

A questionnaire on medical history, current medication use (frequency and dosage), health status and attitude, selected questions on dietary, and physical activity was filled out by all study participants. The following classification was chosen to describe the physical activity behaviour: "predominantly active" (>2 ½ h/week of moderate-intensity or >1 ¼ h/week of vigorous-intensity exercise); "predominantly sedentary" (<2 ½ h/week of moderate-intensity or <1 ¼ h/week of vigorous-intensity exercise); or "regular exercise" (approximately 2 ½ h/week of moderate-intensity or 1 ¼ h/week of vigorous-intensity exercise).

Alcohol consumption was assessed using 3-day food recalls (see above). The maximum acceptable level for women and men was set at 10 g and 20 g of pure alcohol per day, respectively, according to [22]. To characterise their "attitudes to health", subjects were asked whether they expected their health to get worse. Subjects were classified as having an "optimistic self-perception of ageing" or a "pessimistic self-perception of ageing". The exact use of medical drugs was recorded using a specific case report form questionnaire. Within this questionnaire the frequency of use and daily dose were recorded in a free-text response.

Anthropometric and body composition measurements

Height was measured using a stadiometer (Seca GmbH & Co. KG, Hamburg, Germany). Waist circumference (WC) was measured between the lowest rib and the highest hip bone at the narrowest part of the midsection using a

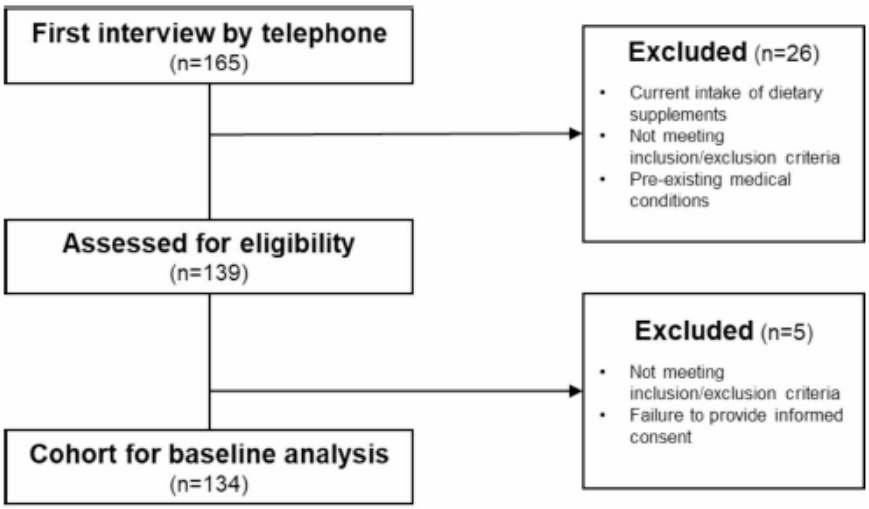

Fig. 1 Flow chart of study population

tape measure. Body weight was measured digitally (Seca GmbH & Co. KG, Hamburg, Germany) to the nearest 0.1 kg (lightly dressed, without shoes). The body composition markers fat mass (FM), lean body mass (BLM), total body water (TBW) and phase angle (PA) were analysed using an 8-point bioelectrical impedance analyser (BIA, mBCA525, Seca Company, Hamburg, Germany). For the measurements, participants were instructed to urinate and remove all jewellery before the examination. Subjects then had to lie down on a stretcher and rest for about 5 min to ensure a balanced distribution of body fluids. All measurements were taken by trained nutritionists of the Institute.

Blood sampling and blood pressure measurement

After an overnight fast (≥12 h fasting period), blood samples were collected by a physician between 08:00 and 11:00 a.m. Blood samples were taken by venipuncture from an arm vein using multifly needles (Sarstedt, Nümbrecht, Germany) into serum or EDTA monovettes (Sarstedt). All samples were stored at − 5 °C and shipped to external laboratories on the same day. Blood pressure was measured by the physician in the sitting position after a resting period of 3–5 min on both upper arms above the elbow.

Biochemical analysis of 25-(OH)D, holoTC and RBC folate

Serum 25-(OH)D was measured in duplicate at SYNLAB MVZ (Leinfelden, Germany) using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS, Recipe, Munich, Germany). For serum 25-(OH)D analysis, mean recoveries were obtained between 85 and 104% with a within-assay coefficient of variation of 2.8% and a limit of detection (LOD) of 1.61 nmol/L and a limit of quantification (LOQ) of 5.4 nmol/L. Serum holoTC was determined using electrochemiluminescence immunoassay (ECLIA) on cobas® test systems (Roche Diagnostics GmbH, Mannheim, Germany) as previously described [15, 23]. For holoTC, mean recoveries were obtained between 88 and 106% with a within-assay coefficient of variation of 3.4% and a limit of detection (LOD) of 3.0 pmol/L and a limit of quantification (LOQ) of 5.0 pmol/L. RBC folate was analysed using ECLIA on Immulite 2000 analyser series (Diagnostic Products Corporation, Los Angeles, USA) with a mean recoveries between 98 and 104%, analytical sensitivity of 1.8 nmol/L and within-assay coefficient of variation of 4.5%.

Concentrations of vitamins are reported in nmol/L in case of RBC folate and 25-(OH)D and in pmol/L in case of holoTC concentrations. Creatinine concentrations are reportd in µmol/l and CRP concentrations in mg/dL. All numbers were rounded to three significant digits.

Reference ranges

In agreement with the recommendations from the NAM and the EFSA the cut-off for serum 25-(OH)D concentrations with >50 nmol/L, as indicative for a "sufficient" vitamin D status, was applied [7, 8]. 25-(OH)D concentrations between 25 - <50 nmol/L were classified as "insufficient" and concentrations<25 nmol/L as "deficient" according to the classification of numerous recent publications [6, 24–26]. For "cobalamin deficiency", a holoTC concentration cut-off of <50 pmol/L was applied [27]. Reference concentrations for RBC folate are highly dependent on the laboratory-specific assay used. With the RBC folate method used by SYNLAB MVZ (Leinfelden, Germany), the reference range of 570–1810 nmol/L was specified. Thus, RBC folate concentrations of <570 nmol/L indicate a "folate deficiency" in the current study population.

Data analysis and statistical methods

All analysis were performed using SPSS statistical software (version 28.0; SPSS, Chicago, IL, USA). The Shapiro-Wilk test was used to test the normal distribution of 25-(OH)D, holoTC and RBC folate concentrations. In addition, quantile-quantile plots were generated for visual inspection. In case of absence, log transformation was performed to obtain a normal distribution. Multiple linear regression models were used to examine associations between concentrations of markers of nutrient status and age and food group intakes. Only food groups considered to be relevant sources of vitamin D, cobalamin and folate were included in the regression analysis. Model 1 represents unadjusted regression analysis and model 2 was adjusted for age and sex and model 3 was fully adjusted for total energy intake, age, sex, body weight, BMI, WC and creatinine concentrations. Statistical significance was set at the level of 0.05.

Results

Baseline characteristics

In total, 134 elderly people were included in the study (Table 1). The age ranged from 70 years up to 100 years at the timepoint of examination with a mean age of 75.8±4.5 years. Almost the same number of subjects lived alone or within a partnership at home (49.4% vs. 50.6%).

Mean body weight, BMI, WC, and body composition markers were within the physiologically range for healthy elderly subjects. Mean serum creatinine concentrations of women (70.4±8.8 µmol/l) and men (88.0±17.7 µmol/l) were within the laboratory specific reference ranges (women: 44.9–83.6 µmol/l; men: 59.0-103 µmol/l). Mean plasma CRP concentrations were also in a very low range.

The cohort was characterised by a high level of education (54% high education level and 15% low education

Table 1 Anthropometric and demographic characteristics, lifestyle and health behaviour of the study population

	Total n = 134	Female n = 97	Male n = 37
	Mean ± SD	**Mean ± SD**	**Mean ± SD**
Anthropometrics			
Weight [kg]	67.4 ± 13.1	67.4 ± 13.1	78.0 ± 12.7
BMI [kg/m²]	25.7 ± 4.6	25.6 ± 5.0	25.8 ± 3.1
WC [cm]	92.6 ± 12.2	90.4 ± 12.4	98.2 ± 9.9
FM [%]	35.6 ± 8.9	38.6 ± 7.9	27.8 ± 6.4
BLM [%]	64.4 ± 9.0	61.4 ± 7.9	72.1 ± 6.4
TBW [L]	33.6 ± 7.3	30.7 ± 5.6	41.2 ± 5.5
PA [°]	4.9 ± 0.5	4.8 ± 0.5	5.1 ± 0.6
Creatinine [µmol/L]	79.2 ± 17.6	70.4 ± 8.8	88.0 ± 17.7
CRP [mg//dL]	2.2 ± 0.9	2.3 ± 0.9	1.9 ± 1.1
	n (%)	**n (%)**	**n (%)**
Age groups			
70–74 years	61 (46)	50 (52)	11 (30)
75–79 years	51 (38)	35 (36)	16 (43)
≥ 80 years	22 (16)	12 (12)	10 (27)
Family status			
Living alone	65 (49)	59 (62)	6 (16)
Living with a partner	68 (51)	37 (38)	31 (84)
Education level			
Low education	20 (15)	16 (17)	4 (11)
Middle education	41 (31)	34 (35)	7 (19)
High education	72 (54)	46 (48)	26 (70)
Medical drug intake[a]			
No intake	30 (21)	24 (25)	6 (16)
Antihypertensive drug	72 (53)	49 (51)	23 (62)
Statins	25 (18)	10 (10)	15 (41)
Proton-pump inhibitor	9 (7)	6 (6)	3 (8)
Polypharmacy	16 (12)	8 (8)	8 (22)
Usual diet			
Omnivor	114 (87)	80 (81)	34 (94)
Vegetarian	17 (13)	15 (19)	2 (6)
Physical activity[b]			
Predominantly active	25 (19)	20 (21)	5 (14)
Predominantly sendentary	13 (10)	10 (10)	3 (8)
Regular basis movement	96 (71)	67 (69)	29 (78)
Smoking status			
Current smoker	3 (4)	3 (5)	0 (-)
Previous smoker[c]	60 (46)	43 (46)	17 (49)
Never smoke	67 (50)	48 (49)	19 (51)
Alcohol use[d]			
Abstinent	33 (25)	25 (26)	8 (22)
< maximum acceptable level	62 (46)	45 (46)	17 (46)
> maximum acceptable level	39 (29)	27 (28)	12 (32)
Attitudes to health			
Optimistic self-perception of ageing	85 (66)	61 (67)	24 (65)
Pessimistic self-perception of ageing	43 (34)	30 (33)	13 (35)

BMI: body mass index; WC: waist circumference; FM: fat mass; BLM: body lean mass; TBW: total body water; PA: phase angle. [a] Physical activity: predominantly active > 2½ h/week of moderate-intensity or > 1¼ h/week of vigorous-intensity exercise; predominantly sedentary < 2½ h/week of moderate-intensity or < 1¼ h/week of vigorous-intensity exercise or regular exercise approximately 2½ h/week of moderate-intensity or 1¼ h/week of vigorous-intensity exercise. [b] Previous smoker: at least one year without smoking. [c] Maximum acceptable level of alcohol use: women: 10 g/day; men: 20 g/day according to [29]. [d] Multiple answers possible. Polypharmacy: ≥5 medical drugs at same time

level) compared to the generel German population aged 65–80 years (14% high education level and 56% low education level) [28].

21% of subjects reported not taking any medication, but 12% had polyvalent medication use (≥5 medications at the same time).

Table 2 Blood concentrations of holotranscobalamin [holoTC], red blood cell [RBC] folate and 25-hydroxycholecalciferol [25-(OH)D]

	Total n = 134	Female n = 97	Male n = 37
	Mean ± SD *n (%)*	Mean ± SD *n (%)*	Mean ± SD *n (%)*
HoloTC [pmol/L]	88.9 ± 33.7	94.8 ± 34.6	73.6 ± 25.6
Deficiency [< 50 pmol/L]	*16 (12)*	*8 (8)*	*8 (22)*
RBC folate [nmol/L]	831 ± 244	845 ± 256	795 ± 210
Deficiency [< 570 nmol/L]	*13 (10)*	*9 (10)*	*4 (12)*
25-(OH)D [nmol/L]	85.1 ± 26.0	85.9 ± 26.8	83.1 ± 23.8
Deficiency [≤ 25 nmol/L]	*2 (1)*	*1 (1)*	*1 (3)*
Insufficiency [25 - < 50 nmol/L]	*8 (6)*	*7 (7)*	*1 (3)*
Sufficiency [≥ 50 nmol/L]	*124 (93)*	*89 (92)*	*35 (94)*

Table 4 Energy-adjusted dietary food group intake calculated via 3-day food recalls

	Total n = 134	Female n = 97	Male n = 37
	Mean ± SD	Mean ± SD	Mean ± SD
Food group intake			
Fruits [g/day]	188 ± 127	192 ± 127	177 ± 124
Vegetables [g/day]	247 ± 148	255 ± 140	225 ± 166
Seeds and nuts [g/day]	14.1 ± 19.4	15.9 ± 20.4	9.26 ± 15.7
Fish [g/day]	33.9 ± 36.0	35.0 ± 38.4	30.9 ± 36.0
Milk/ dairy products [g/day]	154 ± 116	162 ± 100	132 ± 149
Meat, eggs, meat products [g/day]	82.9 ± 62.3	77.1 ± 59.1	98.7 ± 69.7
Grains and bread [g/day]	118 ± 54.1	110 ± 52.7	137 ± 54.1

The energy adjustment was performed using the residuum method, as previously described by Willet et al. [21]

The majority of the subjects were omnivores. 13% of the subjects reported being vegetarians.

Only 10% of the subjects described their physical activity as predominantly sedentary and more than 95% of the subjects were current non-smokers. In addition, about 70% of this cohort reported no alcohol consumption or alcohol consumption below the maximum acceptable level. Finally, participants' attitudes to health were predominantly optimistic, with 66% feeling positive about their future health.

Vitamin D intake and 25-(OH)D concentrations

The dietary intake of vitamin D was low, with a mean ± SD of 4.1 ± 5.0 µg/day in the entire study group. The serum 25-(OH)D concentration of the entire study population was 85.1 ± 26.0 nmol/L (Table 2). The majority of study subjects had 25-(OH)D concentrations ≥ 50 nmol/L, indicating a sufficient vitamin D status (93%). Less than 10% of the participants in this study had an inadequate or deficient vitamin D status during the season of maximum UVB radiation. Using linear regression analyses, we found no significant association between vitamin D intake and serum 25-(OH)D concentration (Table 3).

Cobalamin intake and holoTC concentrations

The dietary intake of cobalamin in the entire study population was 4.0 ± 2.1 µg/day. The holoTC concentration of the entire study population was 88.9 ± 33.7 pmol/L (Table 2). The prevalence of cobalamin deficiency was low in women but relatively high in men. Only 8% of the women had a deficient cobalamin status compared to 22% of the men. The intake of cobalamin was not significantly associated with holoTC concentrations (Table 3).

Folate intake and RBC folate concentrations

The dietary intake of folate equivalents was 251 ± 95.6 µg/day in the total study population. The RBC folate concentration of the entire study population was 831 ± 244 nmol/L (Table 2). Women had slightly higher RBC folate concentrations than men (845 ± 256 nmol/L vs. 795 ± 210 nmol/L). The prevalence of folate deficiency in the entire group was low (10%), with minor differences between the sexes. Folate equivalent intake was significantly associated with RBC folate concentrations in a fully adjusted model (Table 3, Model 3, $p = 0.002$).

Association between dietary food group intake and vitamin status markers

Consumption of milk and dairy products (Table 4) was significantly associated with holoTC concentrations in the unadjusted ($p < 0.001$) and fully adjusted ($p < 0.001$) model (Table 5). Similarly, vegetable intake (Table 4) was significantly associated with RBC folate concentrations in the unadjusted ($p = 0.005$) and the sex and age-adjusted model ($p = 0.044$), but not in the fully adjusted model ($p = 0.268$). For all other food groups, no associations with the vitamin status markers were found.

Table 3 Association of dietary cobalamin, folic acid and vitamin D intake and vitamin status markers

	Beta-coeff. Model 1	p-value Model 1	Beta-coeff. Model 2	p-value Model 2	Beta-coeff. Model 3	p-value Model 3
HoloTC	0.024	0.151	0.073	0.188	0.095	0.243
RBC folate	0.001	0.062	0.013	0.057	0.057	**0.002**
25-(OH)D	0.001	0.716	0.002	0.679	0.062	0.237

Model 1: Unadjusted using cobalamin intake as an independent variable for holoTC, folic acid intake as an independent variable for RBC folate and vitamin D as an independent variable for 25-(OH)D. Model 2: Adjusted for age and sex. Model 3: Adjusted for age, sex, energy intake, body weight, BMI, WC and creatinine

Table 5 Association of food group intake and vitamin status markers

	Beta-coeff. Model 1	p-value Model 1	Beta-coeff. Model 2	p-value Model 2	Beta-coeff. Model 3	p-value Model 3
HoloTC						
Milk and dairy products	0.235	**<0.001**	0.148	**<0.001**	0.173	**<0.001**
Meat, eggs, meat products	0.001	0.236	0.054	0.537	0.052	0.675
RBC folate						
Fruits	0.222	0.300	0.011	0.590	0.041	0.865
Vegetables	0.498	**0.005**	0.053	**0.044**	0.020	0.268
Seed and nuts	0.341	0.135	0.020	0.179	0.016	0.605
Grains and bread	0.002	0.961	0.018	0.728	0.052	0.949
25-(OH)D						
Fish	0.017	0.787	0.008	0.545	0.031	0.172

Model 1: Unadjusted. Modell 2: Adjusted for age and sex. Modell 3: Adjusted for age, sex, energy intake, body weight, BMI, WC and creatinine

Discussion

The aim of the present study was to assess vitamin D, cobalamin, and folate status in unsupplemented, healthy, independently living, active elderly people aged ≥ 70 years.

Although we expected a better status of these nutrients compared to the general population, we were surprised by the very low prevalence of vitamin D, cobalamin and folate deficiencies. Only the male subjects showed a slightly higher prevalence of cobalamin deficiency. However, since the number of men in the study was very small, this result should be treated with caution. Comparable studies in healthy subjects aged ≥ 70 years using tissue markers such as holoTC and RBC folate are rare. In addition, several of these studies included cohorts with a high prevalence of regular use of supplements or fortified foods in countries where this is applicable, which is in contrast to our cohort of unsupplemented individuals.

Vitamin D status

The prevalence of vitamin D deficiency has been investigated in many epidemiologic studies in Europe, with 25-(OH)D being the most commonly used marker [6, 30, 31]. The studies differed in the analytical methods used to investigate serum 25-(OH)D concentrations, with the LC/MS-MS technique being considered the gold standard compared to the chemiluminescent microparticle immunoassay (CLIA). In our study, serum was measured in duplicate by LC/MS-MS, so the results should be analytically accurate.

Several epidemiological studies have shown that the prevalence of 25-(OH)D concentrations < 50 nmol/L is between 30% and 80% worldwide at all ages [32]. Previous observations examining vitamin D status, particularly in older adults ranged from 16 to 79%, depending on season and sex [5, 6, 33, 34]. In the KORA Age Study [33], a cross-sectional study in southern Germany (age range 65 to 93 years, n = 1,079), the prevalence of vitamin D insufficiency was 52%. In comparison, the prevalence of vitamin D insufficiency in the present cohort was low at less than 10%. Our results are also in contrast with data from the German Nationwide Nutrition Survey 1 (DEGS1), which included 6,995 individuals of all ages and reported a high prevalence of vitamin D insufficiency during summer time. Specifically, about half of the adults of both sexes had a vitamin D insufficiency [22]. It should be noted that serum 25-(OH)D in the DEGS1 study was analysed by the CLIA method, which has been described to yield falsely low levels of 25-(OH)D compared to the LC-MS/MS method as used in our study [35]. Under this assumption, the difference in 25-(OH)D concentrations to our data may be reduced. Klenk et al. [5] also observed that the prevalence of 25-(OH)D concentrations < 50 nmol/L in a population of elderly subjects (≥ 65 years, not taking supplements, measured in August) in southern Germany was 16.1% (mean 25-(OH)D: 77.6 nmol/L), which is in line with our results.

The present cohort was characterised by a high level of physical activity, mainly outdoors during the COVID-19 pandemic, resulting in high exposure to UVB radiation, which is the strongest factor influencing the vitamin D status [22, 36, 37]. In addition, the population had a low average BMI and body fat percentage compared to those reported in the DEGS1 study [38]. Therefore, it's important to emphasise that this sample size is not representative of the entire elderly population in Germany.

Dietary sources usually cover up only 10–20% of the vitamin D requirement and therefore do not significantly influence the vitamin D status [39, 40]. This is also evident in our study, where we did not find significant associations between vitamin D intake and 25-(OH)D concentrations. However, a Dutch study as part of the B-PROF trial in 2,530 people aged ≥ 65 years showed a significant association between vitamin D intake and 25-(OH)D concentrations during the summer period [41]. Within a dose-response relationship, the authors suggest a 1 nmol/L increase in 25-(OH)D concentrations with each unit increase in vitamin D intake.

Cobalamin status

HoloTC reflects long-term cobalamin intake and is considered the most valid marker to assess cobalamin status, especially in older individuals (>50 years) [15]. We observed a prevalence of cobalamin deficiency (12%) in the entire study population. Especially men showed a high prevalence of cobalamin deficiency with 22%. The sex differences in cobalamin deficiency are consistent with previous observations [42, 43]. In the National Health and Nutrition Examination Survey (NHANES) survey of 1,770 elderly subjects in the USA, men had a significantly higher risk of cobalamin deficiency than women [44].

Comparable studies using holoTC as a marker to assess cobalamin status in the elderly people are rare [43, 45, 46]. In a Swiss cohort of unsupplemented subjects, mean holoTC concentrations were significantly lower in older subjects (60–69 years: 52.3 pmol/L, 70–79 years: 54.1 pmol/L, ≥80 years: 51.8 pmol/L) compared to our cohort with a mean concentration of 88.9 pmol/L [43]. There were significantly more women than men in our cohort, which means that the mean holoTC concentration is higher than in a "sex-balanced" cohort such as the Swiss cohort. However, even among the men in our cohort, the holoTC concentrations were significantly higher than those in the Swiss cohort. In an Irish cohort of elderly subjects (mean age 72.8 years, 35% men, not taking cobalamin supplements), the mean holoTC concentration (62.7 pmol/L) was also significantly lower compared than in our cohort [46]. The authors showed that the use of proton-pump inhibitors and the presence of atrophic gastritis led to significantly lower holoTC concentrations [46]. In our study, the use of proton-pump inhibitors was quite low (7%). Furthermore, male participants in our cohort had a higher prevalence of medical drug use, which may cause cobalamin malabsorption [13]. In addition, the frequency of medical drug use, especially non-steroidal anti-inflammatory drugs, may be crucial for mucosal damage and consequently reduced availability of food-bound cobalamin [47, 48].

Milk and dairy products and meat and meat products are food groups considered to be good sources of cobalamin. As expected, intake of milk and dairy products was significantly associated with holoTC concentrations and subjects with deficient cobalamin status consumed significantly less milk and dairy products than subjects with sufficient cobalamin status (data not shown). However, this finding cannot explain the overall good cobalamin status, because the subjects consumed less milk and dairy products (women: 162 g/day; men: 132 g/day) than the average German population aged 65–80 years (women: 210 g/day; men: 223 g/day) [16]. In contrast, meat consumption in our cohort (women: 77 g/day, men: 89 g/day) was much higher than in the German population (65–80 years, women: 46 g/day; men: 79 g/day). Surprisingly, no associations were found between holoTC concentrations and meat consumption, and in particular, the high meat consumption among male subjects in this cohort was contrasted with to the significantly higher prevalence of cobalamin deficiency in men.

Folate status

In contrast to serum folate, which is subject to large fluctuations depending on acute dietary intake, RBC folate reflects long-term folate supply and is considered the most reliable marker of the folate status [49, 50]. We observed a predominantly sufficient folate status in the present cohort of elderly people. Comparison with the results of previous studies using the same tissue markers is limited by the fact that subjects in these studies mainly supplemented B vitamins. Folate fortification is not mandatory in Germany, but it is in many other countries. Pfeiffer et al. [44] evaluated RBC folate concentrations in older subjects (≥60 years) of the NHANES cohort before (1988–1994) and after the start of mandatory folate fortification in the USA (1999–2010). The prevalence of folate deficiency was similarly low before (2.1%) and after folate fortification (0.1%).

The adequate folate status in our cohort may be explained by the high intake of vegetables, which are considered to be a good source of folate. With a vegetable consumption of 255 g/day (women) and 222 g/day (men), the subjects in our cohort consumed almost twice the average of the German population aged 65–80 years (women: 128 g/day; men: 123 g/day) [16]. As expected, vegetable consumption was significantly associated with RBC folate concentration. Öhrvik et al. [51] also observed an association between vegetable consumption and RBC folate concentration in a cohort of adults (45–80 years, 46% women), 98% of whom had an adequate folate status.

The use of proton pump inhibitors and regular use of metformin have been described to negatively affect folate status [52]. In our population, the use of these drugs was very low or absent. Smoking [53], physical inactivity and a BMI ≥ 30 kg/m^2 [52] also influence the folate status. However, our subjects were almost exclusively non-smokers, predominantly active and had an age-appropriate BMI. Taken together, these reasons may explain the overall sufficient folate status.

Strengths and limitations

The strength of our study was its straightforward design with well-characterised subjects. In addition, we used state-of-the-art analytical parameters and methods to measure the vitamin D, folate, and cobalamin status. For example, 25-(OH)D concentrations were analysed by LC-MS/MS, which is considered as the gold standard method. Folate status was assessed using RBC folate,

which is the most sensitive marker for assessing the body's' folate status. We also used holoTC, which is the first-line cobalamin marker in populations over 50 years of age [15].

The study has several limitations. The study has a relatively small sample size. As the study cohort consisted mainly of highly educated, active and health-conscious individuals who were willing to participate in a clinical study, the results cannot be extrapolated to the average community-dwelling elderly population in Germany, which does not have the same health-consciousness and physical activity.

The study was conducted during the summer season in Germany, reflecting a favourable situation concerning UVB-radiation and endogenous vitamin D synthesis. In addition, the study was conducted in the midst of the COVID-19 pandemic, when indoor gatherings were prohibited, creating conditions that make comparisons with other studies difficult.

The evaluation of nutrient intake data from food records generally has some bias. Alcohol intake was measured only by 3-day food records and not by food frequency questionnaires. Consumption of fortified products was not asked separately in a validated questionnaire. However, in Germany, food is only occasionally fortified with folic acid compared to other countries, and no significant influence of fortified products can be assumed. When comparing the food group intakes of our population with the average German population [16], it should be noted that the representative reference data were collected over a period of 4 weeks using different survey methods (e.g. food frequency questionnaires, dietary history interviews, weighing records), whereas our data were collected by 3-day food recalls only.

The metabolic markers homocysteine, methylmalonic acid and the aggregated marker 4cB12 are used in clinical trials to assess the cobalamin and folate status [54]. However, these markers may be influenced by impaired renal function or deficiencies of other B vitamins involved in homocysteine metabolism. Finally, we don't have information on the prevalence of infection with *Helicobacter pylori* infection or atrophic gastritis in our cohort, which may predict dietary cobalamin malabsorption. However, cobalamin deficiency was quite low and the prevalence of atrophic gastritis is rather high in Germany [55].

Conclusion

Overall, we observed a low prevalence of vitamin D, cobalamin and folate deficiency in a cohort of individuals aged ≥ 70 years, characterised by high levels of education, physical activity, and health awareness. However, despite a healthy and active lifestyle, a significant proportion of male subjects did not achieve adequate concentrations of holoTC or RBC folate. The latter result must be treated with caution as the number of men in the study cohort was very small. The consolidation of these findings needs to be investigated in further studies, which should explicitly focus on subjects of advanced age > 80 years. In addition, studies are needed to identify lifestyle and dietary patterns that may predict adequate nutrient status in older people and ensure healthy ageing.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12877-023-04391-2.

Supplementary Material 1

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Author contributions

FK: Data acquisition, evaluation and curation, writing-original draft preparation; JPS:Data evaluation, writing, reviewing and editing; AH: Conceptualization and studydesign, methodology, reviewing and editing, supervision; All authors have read andagreed to the submitted version of the manuscript.

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Data Availability

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The ethic committee of the medical chamber of Lower Saxony (Hannover, Germany) approved all procedures. Written informed consent was obtained from all participants prior to their enrolment.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interests.

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Publisher's Note

3. Paper II

A micronutrient supplement modulates homocysteine levels regardless of vitamin B biostatus in elderly subjects

Authors: Kerlikowsky F, Marija Savic-Hartwig, Edda van de Flierdt, Andreas Hahn, and Jan Philipp Schuchardt

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Original Communication

A micronutrient supplement modulates homocysteine levels regardless of vitamin B biostatus in elderly subjects

Marija Savic-Hartwig, Felix Kerlikowsky, Edda van de Flierdt, Andreas Hahn, and Jan Philipp Schuchardt

Institute of Food Science and Human Nutrition, Leibniz University Hannover, Germany

Abstract: Elevated homocysteine (Hcy) levels (≥15 µmol/L) in the elderly are frequently associated with a higher risk of cardiovascular disease and cognitive decline. Several studies have already shown an Hcy-lowering effect of B vitamin supplementation in cohorts deficient in these nutrients. The aim of this randomized, double-blinded 12-week intervention study was to investigate whether Hcy levels in healthy elderly subjects (75.4±4.5 years, n=133) could be lowered with a micronutrient supplement (i.e., 400 µg folic acid, 100 µg cobalamin). Difference in mean initial Hcy levels between intervention (17.6+7.1 µmol/l, n=65) and placebo group (18.9+6.1 µmol/l, n=68) was not significant. The prevalence of cobalamin and folate deficiency in the total study population was low: 27% had serum-cobalamin levels ≤150 pmol/L, 12% holo-transcobalamin (Holo-TC) levels ≤50 pmol/L, 13% low cobalamin status using the aggregated cobalamin marker 4cB12 and 10% red blood cell (RBC) folate ≤570 nmol/L. Nevertheless, the treated subjects still showed improved cobalamin and folate biostatus (serum cobalamin Δt_{12}-t_0: 63+48 pmol/l ; Holo-TC Δt_{12}-t_0: 17+19 pmol/l ; RBC folate Δt_{12}-t_0: 326+253 nmol/l) and Hcy levels (Δt_{12}-t_0: −3.6+5.7 µmol/l). The effects were statistically significant compared to the placebo group with p=0.005 (serum cobalamin), p=0.021 (Holo-TC), p=0.014 (RBC-folate) and p<0.001 (Hcy). The Hcy-lowering effect was dependent on the initial Hcy levels (p<0.001). Our findings suggest that elevated Hcy levels in elderly subjects can be lowered regardless of the initial cobalamin and folate biostatus.

Keywords: hyperhomocysteinemia, homocysteine, vitamin B12, folate, multivitamin

Background

Homocysteine (Hcy) is an amino acid synthesized as an intermediate product of methionine metabolism. Elevated Hcy levels cause endothelial dysfunction by a) increasing the production of reactive oxygen species [1], b) promoting the oxidation of low-density lipoprotein [2], c) toxic shedding of endothelial cells [3] as well as d) damaging the medial and adventitial layers of the arterial vessel wall [4]. Moreover, Hcy stimulates prothromboplastic activity by increasing platelet aggregation [5], modulates protein function [6], and has both neurotoxic and genotoxic properties [7, 8]. Abnormally high plasma Hcy levels are considered a medical condition called hyperhomocysteinemia [9]. There is no general consensus on the cutoff values for hyperhomocysteinemia [6, 10, 11]. Recent trials have commonly used a cut-off of ≥15 µmol/L [12, 13].

A pathological increase in plasma Hcy levels is considered an independent cardiovascular risk factor [14, 15, 16]. However, studies on B-vitamin intervention for lowering plasma Hcy levels showed no advantages for secondary prevention with regard to cardiovascular disease [17]. Nevertheless, elevated plasma Hcy levels are viewed as componential triggers for a variety of multifactorial diseases, such as atherosclerosis [7], congestive heart failure and illnesses that are typical in the elderly, such as dementia (in particular Alzheimer's disease) [18, 19], age-related macular degeneration and hearing loss [3]. There is also an association with osteoporosis [20, 21, 22]. Altogether, elevated Hcy levels are associated with more than 100 health conditions [23].

Hyperhomocysteinemia is mainly caused by reduced activity of key enzymes involved in Hcy metabolism due to dietary B vitamin deficiencies [24]. In particular, cobalamin (vitamin B12), folate and pyridoxine (vitamin B6) play major roles in Hcy metabolism [25]. Hence, plasma Hcy levels are viewed as an additional functional marker of folate and cobalamin deficiencies [26, 27]. Previous studies have shown that deficiencies in B vitamins and high plasma

Hcy levels are negatively associated and frequent in the elderly [3, 28, 29, 30]. Another cause of disturbed Hcy metabolism is functional gene polymorphisms [24].

Plasma Hcy levels are higher in males compared than in women and increase with age [31]. Therefore, elderly people are at risk of hyperhomocysteinemia [28, 32]. An increase in Hcy levels is possible due to an unbalanced diet or malnutrition, which is frequent in elderly people [33]. On the other hand, an age-related malabsorption of nutrients is a major cause of insufficient B vitamin biostatus and the development of hyperhomocysteinemia [34]. In old age, both autoimmune (type A) and bacterial (*H. pylori*) atrophic gastritis (type B) are frequent (up to 30%) and lead to a reduced formation of intrinsic factor (IF), which normally binds cobalamin and to decreases gastric acid secretion (hypochlorhydria), resulting in a decreased release and absorption of cobalamin [34, 35]. Hcy metabolism is also influenced by the intake of certain drugs [36, 37, 38] and impaired kidney function [39, 40].

Numerous interventional studies observed an Hcy-lowering effect after folate and cobalamin supplementation [41, 42, 43, 44]. However, very few studies have been conducted on seniors aged ≥70 years [34, 45]. To assess the relevance of vitamin supplementation, it is important to measure the prevalence of cobalamin and folate deficiencies [46, 47]. Intervention studies often did not report the initial prevalence of cobalamin- and folate deficiency using valid biomarkers reviewed by Olaso-Gonzalez et al. [48]. It is unclear whether elevated Hcy levels can be lowered in elderly people with a sufficient micronutrient biostatus. In addition, the comparison of results from previous studies is complicated because different biomarkers are used to assess cobalamin and folate biostatus [49]. Frequently, blood levels are measured only in the serum. However, red blood cell folate (RBC folate), holotranscobalamin (Holo-TC) and the combined marker 4cB12 are considered more valid for assessing the long-term biostatus of the two B vitamins [50, 51, 52, 53], since serum cobalamin can fluctuate daily and may inadequately represent cobalamin status in tissues [54, 55, 56]. Consistent with this, a recent study by Campos et al. [52] suggested that Holo-TC should be used as a preferred first-line marker for the detection of subclinical cobalamin deficiency in individuals aged ≥50 years.

Hence, in the present study, we aimed to determine and evaluate the effect of multivitamin supplements, including cobalamin and folate, on plasma Hcy levels in healthy elderly subjects aged ≥70 years. This study is part of a larger trial with the overall aim of assessing and improving the status of critical micronutrients in older people and to investigate their impact on a number of health-related biomarkers, including the Hcy level. Folate biostatus was evaluated using RBC folate, while cobalamin biostatus was assessed using serum cobalamin, Holo-TC and 4cB12. In addition, the methylmalonic acid (MMA) concentrations were measured.

Material and methods

Study design and participants

The study was conducted as a single-center, two-armed, double-blinded, and randomized clinical trial at the Institute of Food Science and Human Nutrition, Leibniz University Hannover, Germany according to the guidelines of the Declaration of Helsinki and registered in the German Clinical Register (DRKS00021302).

In total, 133 healthy subjects met all the inclusion criteria (≥70 years, living home dwelling, and independently) and were included in the study (Figure 1). Exclusion criteria were defined as intake of dietary supplements up to three months before the examination, BMI>35 kg/m^2, severe gastrointestinal or cardiovascular diseases, and intake of immunosuppressants or chronic corticosteroids. The participants provided informed consent before enrollment. Subjects were recruited through local press advertisements and announcements in senior network centers and volunteer clubs. Interested subjects were screened for their health status, as well as for the intake of dietary supplements, through a telephone interview (Figure 1).

After controlling for the inclusion and exclusion criteria, the subjects were invited to the institute for examination. Participants completed a questionnaire regarding their medical history, current medical drug intake (frequency and dosage) and health status, and selected questions on general diet and physical activities. Participants described their movement behavior based on the following classification: as "predominantly active" (>2 ½ hours/week movement with middle intensity or >1 ¼ hours/week with high intensity); "predominantly sedentary" (<2 ½ hours/week movement with middle intensity or <1 ¼ hours/week with high intensity) or "regular basis movement" (in approximation to 2 ½ hours/week movement with middle intensity or 1 ¼ hours/week with high intensity).

The examination days included measurement of anthropometric data, including body weight and height (Seca GmbH & Co. KG, Hamburg, Germany), waist and hip circumferences, blood pressure and pulse rate. Consequently, blood pressure and pulse rate were performed after a 5 min rest using volume-plethysmography (Boso ABI-system 100; BOSCH & SOHN, Germany) in the left arm. All the measurements were performed by trained nutritionists. The subjects were asked not to change their diet or physical activity during the intervention. At the final examination, the participants completed a second questionnaire regarding changes in medical drug intake, health status, nutrition,

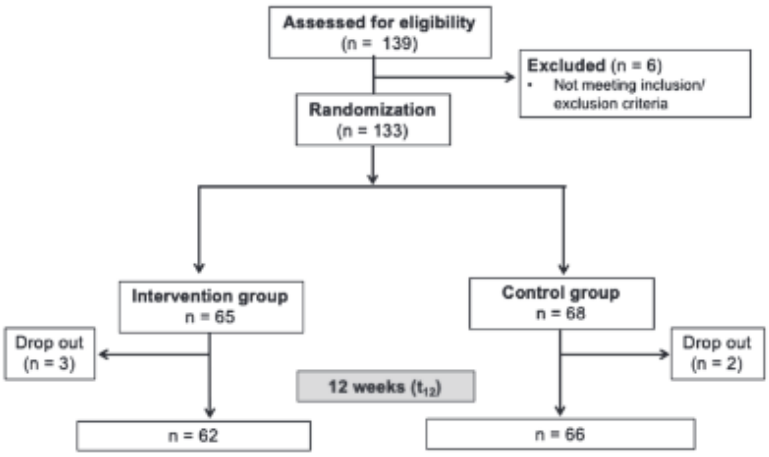

Figure 1. Flow diagram of the study population.

and movement behavior. Subjects reporting major changes were excluded from the analysis.

Supplement

The overall aim of the study was to assess and improve the status of critical nutrients such as numerous vitamins and minerals and to investigate the effects on metabolism, well-being and immune function. As the supply of long-chain omega-3 fatty acids is also unfavorable in elderly people, omega-3 fatty acids have also been supplemented. Accordingly, participants in the intervention group were required to take two different micronutrient supplement capsules per day over the duration of 12 weeks. One capsule contained the following micronutrients: vitamin A:400 µg retinol equivalent (RE), cholecalciferol 50 µg, tocopherol 18 mg (α-TE), vitamin K 30 µg, ascorbic acid 200 mg, thiamine 1.65 mg, riboflavin 2.1 mg, niacin 16 mg niacin equivalent (NE), pyridoxine 2.1 mg, folic acid 400 µg, cobalamin 100 µg, biotin 50 µg, pantothenic acid 6.0 mg, zinc 10 mg, selenium 100 µg, chrome 40 µg, molybdenum 50 µg, iodine 100 µg. The second capsule contained 1.0 g long chain omega-3 fatty acids. Two placebo preparations were administered. The placebo group had to consume one capsule consisting of 500 mg maltodextrin and one capsule with 1.0 g evening primrose oil.

Blood sampling

Blood samples were collected between 08:00 and 11:00 a.m. at baseline and final examination after overnight fasting (minimum 12 h fasting period). If possible, participants were invited to t12 at the same time of the day as for the t0 examination. Blood samples were obtained by venipuncture of the arm vein using Safety-Multifly® needles (Sarstedt, Nümbrecht, Germany) into serum, EDTA, or S-Monovettes® for tHcy (Sarstedt). All samples were stored at approximately 5 °C and transferred to the laboratory on the same day.

Biochemical analyses

All biomarkers were determined in accredited and certified laboratories (LADR Laborärztliche Arbeitsgemeinschaft, Hannover, Germany; SYNLAB MVZ, Leinfelden, Germany). Cobalamin and Holo-TC levels were determined in serum using the electrochemiluminescence immunoassay method (ECLIA) on cobas® test systems (Roche Diagnostics GmbH, Mannheim, Germany) [57, 58].

Liquid chromatography coupled with mass spectrometry (LC-MS/MS) was performed out [59]. Plasma Hcy was determined by high-performance liquid chromatography (HPLC) with a fluorescence detector [60]. Folate in RBCs was analyzed using ECLIA on Immulite 2000 analyzer series (Diagnostic Products Corporation, Los Angeles, USA) in SYNLAB MVZ (Leinfelden, Germany) [61]. Creatinine was determined enzymatically from the plasma samples (Beckman Coulter AU analyzer).

To determine the cobalamin biostatus, the aggregated marker 4cB12 was calculated based on Holo-TC, serum cobalamin, MMA and Hcy levels, according to the following formula [62]:

$$4cB12 = \log 10\left(\frac{\text{HoloTC} \times \text{B12}}{\text{MMA} \times \text{tHcy}}\right) - \left(\frac{3.79}{1+\left(\frac{age}{230}\right)^{2.6}}\right)$$

Holo-TC and serum cobalamin levels were reported in pmol/L and Hcy and MMA levels were standardized to µmol/L.

Reference levels

The following cut-off points were used for cobalamin deficiency: 150 pmol/L for serum cobalamin and 50 pmol/L for Holo-TC, as suggested in recent studies [12, 39, 63]. MMA levels >270 nmol/L [39] und Hcy levels ≥15 µmol/L [9, 12, 64, 65] were considered elevated. In addition, a cut-off of 15 µmol/L is recommended for Hcy analysis methods with HPLC [21], whereas other cut-offs are applied for other methods. The reference levels for RBC folate are highly dependent on the laboratory-specific assay used [66]. According to the manufacturer's instructions (Diagnostic Products Corporation, Los Angeles, USA), a reference range of 570–1810 nmol/L was specified for the RBC folate analysis method carried out. Consequently, RBC folate levels <570 nmol/L indicate folate deficiency in the current study population.

Statistical analysis

Statistical analyses were performed using SPSS software (IBM SPSS Statistics 28.0; Chicago, IL, USA). Continuous variables are shown as mean±standard deviation (SD), while qualitative variables are presented either as absolute or relative frequencies, or only in relative figures. The Shapiro-Wilk test was used to test for normal distribution [67]. In addition, quantile-quantile plots were created for visual inspection. Intention-to-treat analysis was performed to avoid potential bias owing to the exclusion of subjects. The Student's t-test was used to compare variables in the case of normally distributed data. If logarithmic data transformation failed to obtain a normally distributed dataset, the Mann-Whitney U test was performed to test for differences in non-normally distributed data. The chi-squared test was used to determine the distribution of nominal variables between the intervention and placebo groups. Finally, to assess differences between the two groups after the intervention, one-way analysis of variance (ANOVA) was used. To assess the primary intervention effect, the variable Hcy was explored in an analysis of covariance (one-way ANCOVA), controlling for the covariates of age and sex. Linear regression models were used to detect associations between micronutrient biostatus at baseline and the intervention effect, adjusted for age, sex, BMI and creatinine level. For all analyses, statistical significance was set at p levels <0.05.

Results

The baseline characteristics of the study participants are presented in Table 1. In total, 133 home-dwelling elderly participants between 70 and 100 years of age were included in the study. The study population had a higher proportion of women (72.9%). Participants had a mean age of 75.4 ±4.5 years with an average BMI of 25.7±4.6 kg/m^2. Participants were mostly physically active (regular basis movement, 70.1%; predominantly active, 19.7%; predominantly sedentary, 10.2%; data not shown). Moreover, 90.7% of the participants reported a good to excellent health status and less than 4% were current smokers. Noticeable differences between the intervention and placebo groups were detected only in systolic blood pressure (p=0.044).

The evaluation of the questionnaires showed that the participants did not change their nutritional behavior and lifestyle, including movement behavior, over the intervention period.

Levels of serum cobalamin, Holo-TC, RBC folate, MMA and calculated 4cB12

At the baseline examination, serum cobalamin levels did not differ between the intervention and placebo groups (intervention group: 193±70.8 pmol/L; placebo group: 213±111 pmol/L; p=0.496; Table 2). 26.6% of subjects in the intervention group were deficient according to the cut-off level of 150 pmol/L while in the placebo group, the prevalence was 28.4% (Table 3). For Holo-TC, no difference between groups could be observed at baseline (intervention group: 87.3±32.5 pmol/L; placebo group: 91.1±34.9 pmol/L; p=0.572; Table 2). 12.3% of the participants in the intervention group and 11.8% in the placebo group, Holo-TC levels below the cut-off of 50 pmol/L (Table 3). In accordance, Using the aggregated marker 4cB12 showed that the cobalamin biostatus was predominantly adequate (intervention group: 87.9%, placebo group: 85.3%; Table 3). No significant difference was found between the groups at baseline (p=0.661).

Mean RBC folate baseline levels were also above the threshold for deficiency (<570 nmol/L) and did not differ between the groups (intervention group: 792±212 nmol/L; placebo group: 872±265 nmol/L; p=0.067; Table 2). 9.1% of subjects in the intervention group and 10.3% of the placebo group, RBC folate levels that can be classified as folate deficient (Table 3). With regard to MMA, no statistically significant differences between the two groups were observed at baseline. Mean MMA levels were below the cut-off level of 270 nmol/L (intervention group: 259.9±85.0 nmol/L; placebo group: 286.3±111.2 nmol/L; p=0.469; Table 2). However, 30.8% of subjects in the intervention group and 44.1% in the placebo group had elevated MMA levels (Table 3).

Cobalamin and Holo-TC levels significantly increased in the intervention group after 12 weeks of micronutrient supplementation, whereas no changes were observed in the placebo group (Figure 2, Table 2). Moreover, the prevalence

Table 1. Baseline characterization of the study population

	Total n=133 n (%)	Intervention group n=65 n (%)	Placebo group n=68 n (%)	p
Gender				
Female	97 (72.9)	47 (72.3)	50 (73.5)	0.874
Male	36 (27.1)	18 (27.7)	18 (26.5)	
Age groups				
70–74 y	61 (45.9)	34 (52.3)	27 (39.7)	
75–79 y	50 (37.6)	22 (33.8)	28 (41.2)	0.336
≥80 y	22 (16.5)	9 (13.9)	13 (19.1)	
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	
Age (y)	75.4 ± 4.5	75.8 ± 5.0	76.1 ± 4.0	0.098[a]
Weight (kg)	70.3 ± 13.9	70.4 ± 13.8	70.3 ± 14.1	0.995[a]
Body mass index (kg/m^2)	25.7 ± 4.6	25.5 ± 4.1	25.8 ± 5.0	0.868[a]
Waist circumference (cm)	92.6 ± 12.3	92.9 ± 11.7	92.4 ± 12.9	0.812[b]
Waist-hip ratio	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.568[a]
Blood pressure (mmHg), n=132				
Systolic	144 ± 16.6	142 ± 15.8	147 ± 17.0	**0.044*[a]**
Diastolic	84.6 ± 11.0	84.3 ± 9.8	84.9 ± 12.2	0.748[b]
Pulse rate (per minute), n=131	66.7 ± 9.6	65.8 ± 9.6	67.5 ± 9.6	0.372[b]
Hcy (µmol/l)	18.2 ± 6.7	17.6 ± 7.1	18.9 ± 6.1	0.093[a]
70–74 y	17.3 ± 6.5	16.7 ± 6.8	18.0 ± 6.1	0.360[a]
75–79 y	18.0 ± 5.3	17.5 ± 5.2	18.4 ± 5.6	0.755[a]
≥80 y	21.4 ± 6.7	20.7 ± 11.7	21.9 ± 6.8	0.209[a]

Years (y) of homocysteine (Hcy). Group differences were determined using the chi-square test, unless otherwise stated. [a]Mann-Whitney U test, [b]Student's t-test (for independent samples). *Statistically significant differences between groups.

Table 2. Mean levels of Hcy, serum cobalamin and folate biostatus markers before and after the intervention

	Intervention group		Placebo group			
Time	**n**	**Mean ± SD**	**n**	**Mean ± SD**	**Effect size (95% CI)**	**p**
Hcy (µmol/L)						
t0	65	17.6 ± 7.2	68	18.9 ± 6.1		0.067[a]
t12	62	13.6 ± 3.0	66	18.5 ± 5.2	0.101	**<0.001*[b]**
MMA (nmol/L)						
t0	65	260 ± 85.0	68	286 ± 111		0.469[c]
t12	62	241 ± 64.2	66	288 ± 132	0.067	**0.003*[d]**
Serum cobalamin (pmol/L)						
t0	65	193 ± 70.8	68	213 ± 101		0.496[a]
t12	62	240 ± 85.3	66	189 ± 85.8	0.376	**<0.001*[d]**
Holo-TC (pmol/L)						
t0	65	87.3 ± 32.5	68	91.1 ± 34.9		0.572[a]
t12	62	104 ± 31.3	66	87.3 ± 33.6	0.280	**<0.001*[d]**
4cB12						
t0	65	−0.07 ± 0.4	68	0.07 ± 0.5		0.962[a]
t12	62	0.24 ± 0.4	66	−0.14 ± 0.5	0.055	**<0.001*[d]**
RBC folate (nmol/L)						
t0	53[e]	792 ± 212	64[e]	872 ± 265		0.208[a]
t12	53[e]	1124 ± 223	64[e]	870 ± 252	0.391	**<0.001*[d]**

Homocysteine (Hcy), methylmalonic acid (MMA), holo-transcobalamin (Holo-TC), 4cB12 = aggregated marker for cobalamin status calculated based on Holo-TC; serum cobalamin; MMA and Hcy levels, red blood cell folate (RBC folate). P levels reported using [a]Student's t-test (for independent samples), [b]one-way analysis of variance with sex and age as covariates (ANCOVA), [c]Mann-Whitney U Test, [d]one-way ANOVA. *Statistically significant difference between groups. [e]Technically inadequate samples predict a lower number of cases within RBC folate at t0.

Table 3. Prevalence of micronutrient deficiencies and elevated biomarker levels in intervention and placebo groups at baseline and after 12 weeks

	Time	Intervention group %	Placebo group %	p
Hcy				
Elevated (≥15 µmol/L)	t0	60.0	66.2	0.460
	t12	35.5	72.7	**<0.001***
MMA				
Elevated (>270 nmol/L)	t0	30.8	44.1	0.112
	t12	23.0	39.4	**0.046***
Serum cobalamin				
Deficient (≤150 pmol/L)	t0	26.6	28.4	0.818
	t12	12.9	33.8	**0.005***
Holo-TC				
Deficient (<50 pmol/L)	t0	12.1	11.8	0.923
	t12	1.6	10.6	**0.021***
RBC folate				
Deficient (<570 nmol/L)	t0	9.1	10.3	0.948
	t12	0	9.2	**0.014***
4cB12				
Elevated (>1.5)	t0	0	0	/
	t12	0	0	/
Adequate (−0.5 to 1.5)	t0	87.9	85.3	0.661
	t12	98.4	87.9	**0.022***
Low (−1.5 to −0.51)	t0	12.1	14.7	0.661
	t12	1.6	10.6	**0.022***
Possible deficiency (−1.51 to −2.5)	t0	0	0	/
	t12	0	1.5	/
Probable deficiency (<−2.5)	t0	0	0	/
	t12	0	0	/

Homocysteine (Hcy), methylmalonic acid (MMA), holo-transcobalamin (Holo-TC), red blood cell folate (RBC folate) and 4cB12=aggregated marker for cobalamin status calculated based on Holo-TC, serum cobalamin, MMA and Hcy levels. Group differences were assessed using the chi-squared test. *Statistically significant differences within groups.

of low Holo-TC levels decreased in the intervention group from 12.1% to 1.6%. Within the placebo group, we observed a decrease from 14.7% to 10.6% (Table 3).

Similarly, RBC folate levels were significantly increased in the intervention group (p<0.001; Figure 2, Table 2). After 12 weeks of intervention, no subject had folate deficiency based on RBC folate levels (Figure 2), while the mean RBC folate levels even dropped slightly in the placebo group (Table 2).

MMA levels were reduced by 7.4% on average in the intervention group (p=0.003), whereas no changes in the placebo group were observed (Table 2). The change in MMA levels was significantly associated with baseline MMA (p<0.001), Holo-TC (p=0.010) and RBC folate (p=0.018) levels, but not with cobalamin levels (data not shown).

Plasma Hcy levels

At baseline, the mean plasma Hcy level in the study population was 18.2 µmol/L with wide ranges from 8.5 to 51.1 µmol/L (Table 2). The differences in plasma Hcy levels between the intervention and placebo groups were not significant (Figure 3a, Table 2). 63.2% of the total population was hyperhomocysteinemic with plasma Hcy levels ≥15 µmol/L, while no differences between the intervention group (60.0%) and placebo group (66.2%) could be observed (Table 3). Plasma Hcy levels were on average 3.1 µmol/L higher in men than in women (p=0.031) and differed significantly between the age group 70–74 y (17.3 ±6.5 µmol/L) and ≥80 y (21.4±6.7 µmol/L; p=0.025; Table 1). Using adjusted linear regression (adjusted model for sex, BMI and creatinine), plasma Hcy levels were found to be significantly associated with age (p=0.021, beta=0.201; data not shown), serum cobalamin (p=0.036; beta=−0.180) and Holo-TC (p=0.017; beta=−0.210), but not with RBC folate (p=0.411; beta=−0.074; Table E1 in Electronic Supplementary Material 1).

Table 4 displays the micronutrient biostatus at baseline in relation to the normal and elevated plasma Hcy levels. Overall, the prevalence of micronutrient deficiency and increased MMA levels was almost the same in the Hcy-subgroups, except for serum cobalamin. The prevalence of serum cobalamin deficiency was significantly higher in

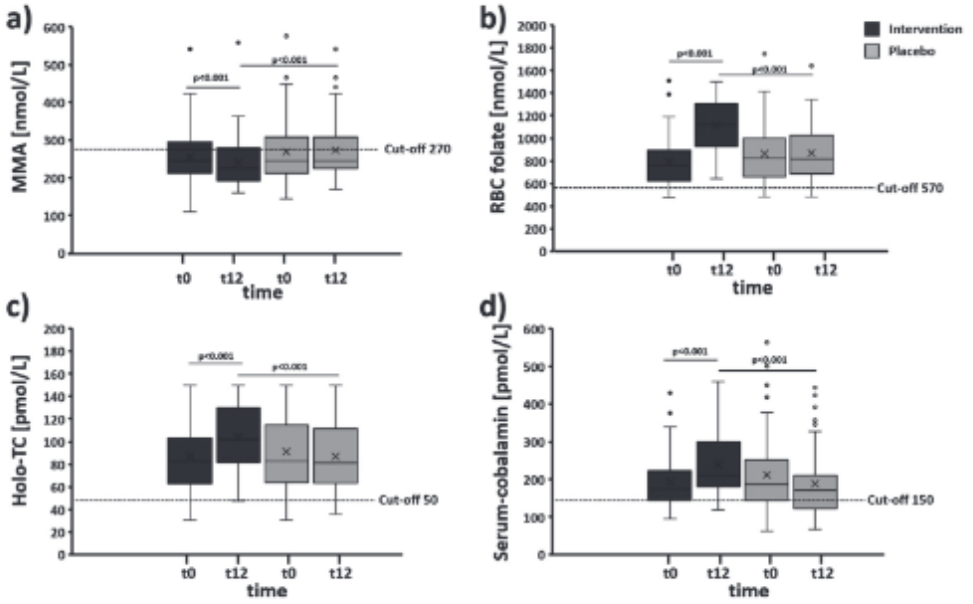

Figure 2. Levels of a) methylmalonic acid (MMA), b) red blood cell folate (RBC folate), c) holo-transcobalamin (Holo-TC), and d) serum cobalamin in the intervention and placebo group initial (t0) and after 12 weeks (t12) of intervention. a) Statistically significant difference between mean t0 and mean t12 MMA levels within the intervention group with $p<0.001$ and significant difference between mean t12 MMA levels of intervention group and mean t12 levels of placebo group with $p=0.007$, b) statistically significant difference between mean t0 and mean t12 RBC folate within the intervention group with $p<0.001$ and significant difference between mean t12 RBC folate of intervention group and mean t12 RBC folate of placebo group with $p=0.001$, c) statistically significant difference between mean t0 and mean t12 Holo-TC within the intervention group with $p<0.001$ and significant difference between mean t12 Holo-TC of intervention group and mean t12 Holo-TC of placebo group with $p=0.001$, d) statistically significant difference between mean t0 and mean t12 serum cobalamin within the intervention group with $p<0.001$ and significant difference between mean t12 serum cobalamin of intervention group and mean t12 serum cobalamin of placebo group with $p=0.001$. x: within the boxplot denotes the mean of the dataset.

subjects with elevated plasma Hcy levels ($p=0.042$). In addition, no significant difference between normal and elevated plasma Hcy levels was observed with respect to medical drug intake ($p=0.668$).

Discussion

In this study, we showed that an intervention with a micronutrient supplement, including cobalamin and folate, over 12 weeks significantly decreased elevated plasma Hcy levels in an elderly population that was mostly well supplied with cobalamin and folate even before intervention. Overall, there is a lack of studies on Hcy reduction with cobalamin and folate supplementation in participants aged ≥70 years. The mean plasma Hcy reduction was −3.6±5.7 µmol/L corresponding to a relative reduction of 23%.

The Hcy-reducing effect of micronutrient supplementation depends on many factors, which complicate the interpretation of intervention studies. Differences in the duration of the intervention time (weeks to months), the composition and dosage of the supplements and the characteristics of the population (e.g., age, baseline Hcy, biostatus of cobalamin, folate and pyridoxine; healthy vs. renal dysfunction) make it difficult to compare the study results and are the cause for varying effect sizes in plasma Hcy reduction. Moreover, the intake of micronutrients via the background diet, including fortified foods, varies significantly between countries. Certain foods in countries such as the US, Canada and South Africa are generally fortified with folate [68]. This makes it difficult to compare study results between different countries and underlines the necessity of measuring vitamin B biostatus with valid markers. All these factors have led to heterogeneous results in previous studies [34, 39]. The decrease in plasma Hcy in response to B vitamin supplementation, therefore, highly varied between 9.8% and 48.6% [48].

The observed mean plasma Hcy reduction of 23% was in line with results of previous studies. The decrease in Hcy with our micronutrient supplement is comparable to our previous work with younger subjects, where the Hcy-lowering effect was also dependent on initial plasma Hcy values [69]. We determined a significant and biologically relevant Hcy reduction in the study cohort with an average relatively good cobalamin and folate biostatus. This did not agree with

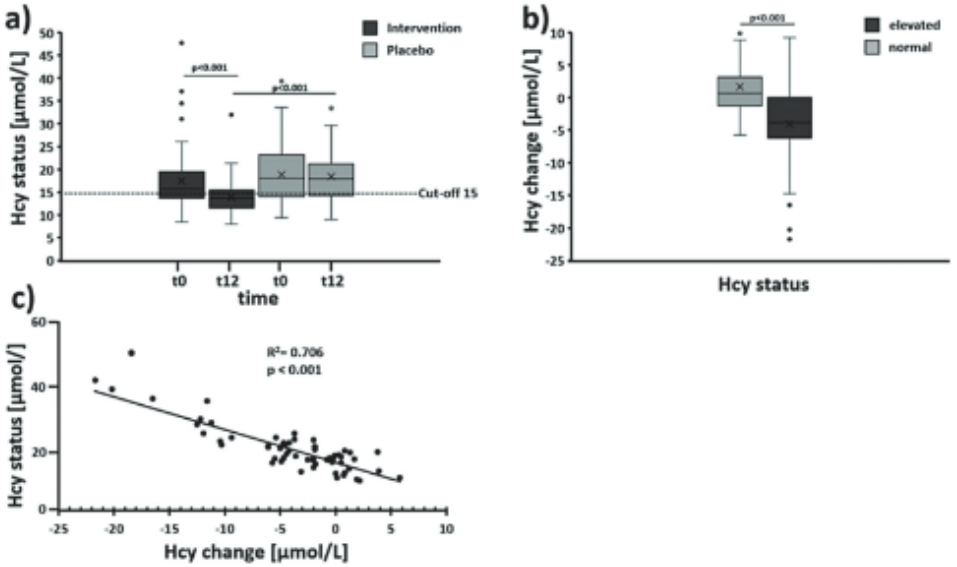

Figure 3. Plasma homocysteine (Hcy) levels in the intervention and placebo group. a) initial (t0) and after 12 weeks of intervention (t12). Statistically significant difference between mean t0 and mean t12 plasma Hcy levels within the intervention group with p<0.001 and significant difference between mean t12 plasma Hcy levels of intervention group and mean t12 plasma Hcy levels of placebo group with p=0.001 b) Change of plasma Hcy levels in subjects with normal (<15 µmol/L) and elevated (>15 µmol/L) plasma Hcy levels at baseline differ statistically significant with p<0.001. c) Linear regression analysis (adjusted model for gender, BMI, creatinine and age) of plasma Hcy level decrease dependent on Hcy levels at baseline (beta=−0.955; r=0.741; p<0.001). x: within the boxplot denotes the mean of the dataset.

the cardinal principle of nutrition that only subjects with an initial deficient status benefit from supplementation [70]. However, previous studies also showed an Hcy-lowering effect in subjects without deficiencies, but these studies failed to analyze valid cobalamin and folate biomarkers, as in this study [71].

The prevalence of cobalamin and folate deficiency in our otherwise healthy study population is in line with the prevalence of vitamin B deficiency in comparable studies with elderly German subjects [45, 72]. In this study, we used long-term biomarkers, in particular, RBC folate, Holo-TC and the aggregated biomarker 4cB12, for the assessment of vitamin B supply, which, to the best of our knowledge, has not been used in any other trial to date. Therefore, there is no comparative data from other studies on the biostatus of Holo-TC and 4cB12. Moreover, many current Hcy-lowering trials failed to assess cobalamin and folate biostatus with valid long-term biomarkers, linked to the prevalence of deficiencies before and after intervention [73, 74, 75, 76].

The biostatus of cobalamin and folate is inversely correlated with Hcy plasma levels [77, 78]. However, we found that the majority of subjects in the present cohort demonstrated elevated plasma Hcy levels despite Holo-TC, 4cB12 and RBC folate levels being within the normal reference range. Of course, it should not be neglected that a significant amount of 12% (based on Holo-TC), 13% (based on 4cB12), or 10% (based on RBC folate) of the subjects had a cobalamin or folate deficiency and elevated Hcy levels. Only a few comparable studies have arrived at similar conclusions. De Koning et al. [73] have shown that healthy elderly people have elevated levels of plasma Hcy and MMA, whereas serum levels of related vitamins are within the normal range. Smith et al. [79] also reported sufficient cobalamin and folate biostatuses in an elderly study population using serum cobalamin and serum folate as biomarkers. After intervention with B vitamins, they found a difference in mean plasma Hcy levels of 31.7% between intervention and placebo.

One potential explanation for the Hcy-lowering effect, despite sufficient cobalamin and folate biostatus, might be age-related impairment in the activity of enzymes [especially methionine synthase or 5-, 10-methylenetetrahydrofolate reductase (MTHFR)] that are involved in the regeneration of Hcy to methionine [33, 80]. Similarly, additional folate may compensate for the restricted availability of 5-methyltetrahydrofolate in subjects with genetic MTHFR polymorphism [81]. Another explanation for the study effects could be that our micronutrient supplement included not only B vitamins but also antioxidants (e.g., ascorbic acid, tocopherol, zinc and selenium), cholecalciferol and omega-3 fatty acid. Plasma Hcy levels were reported to be inversely related to omega-3 supplementation [82]. To date, the physiological background of this

Table 4. Prevalence of cobalamin and folate deficiency, elevated MMA levels, and medical drug intake in subjects with normal and elevated plasma Hcy levels at baseline

	Normal Hcy (<15 µmol/L) n=49	Elevated Hcy (≥15 µmol/L) n=84	p
Serum cobalamin			
Deficient (≤150 pmol/L)	16.3%	32.5%	**0.042***
Holo-TC			
Deficient (<50 pmol/L)	16.3%	9.5%	0.245
4cB12			
Adequate (−0.5 to 1.5)	72.2%	62.0%	0.405
Low (−1.5 to −0.51)	27.8%	38.0%	
RBC folate			
Deficient (<570 nmol/L)	7.5%	3.0%	0.370
MMA			
Elevated (>270 nmol/L)	28.6%	45.3%	0.057
Medical drug intake			
Yes	79.2%	75.9%	0.668
No	20.8%	24.1%	

Homocysteine (Hcy), holo-transcobalamin (Holo-TC), 4cB12=aggregated marker for cobalamin status calculated based on Holo-TC; serum cobalamin; MMA and Hcy levels, red blood cell folate (RBC folate), and methylmalonic acid (MMA). The Hcy subgroups were formed from the total study population. Group differences were assessed using the chi-squared test. *Statistically significant differences between groups.

condition is not fully understood. Omega-3 fatty acids may induce enzymes involved in Hcy metabolism via modulation of gene expression [82]. Dawson et al. [83] reviewed the significant plasma Hcy-lowering effect associated with omega-3 fatty acids, but not all studies showed a positive change in plasma Hcy levels. In principle, this effect was more pronounced when omega-3 fatty acid supplementation was combined with vitamin B supplementation.

Moreover, supplementation with antioxidants could have a beneficial effect on the study outcomes. Breilmann et al. [84] examined the effect of antioxidant supplementation on ascorbic acid, tocopherol and β-carotene on plasma Hcy levels in an aging population. The authors reported that ascorbic acid levels were a relevant predictor of plasma Hcy levels; however, intervention with antioxidants was not associated with Hcy levels. An inverse relationship between antioxidants and elevated Hcy levels has also been reported by Floegel et al. [85]. Racek et al. [86] reported that antioxidant supplementation did not affect Hcy concentrations, but improved antioxidative defense and inhibited peroxidation. Further studies are necessary to clarify the effects of antioxidants on Hcy levels. However, the mechanism of action is unclear.

Several micronutrients are described to improve renal function [87, 88, 89]. Considering that older age is correlated with reduced kidney function, the decrease in Hcy might be partly explained by the regeneration of kidney function through micronutrient supplementation. Considering that the kidney plays an important role in Holo-TC metabolism, improved kidney function leads to reduced biological loss of Holo-TC via the urine [90].

Although Holo-TC and RBC folate are considered reliable biomarkers to reflect the biostatus of micronutrients in tissues [91, 92], there is debate that Holo-TC and RBC folate levels might not reflect the intracellular bioavailability of cobalamin and the metabolically active form of folate (5,10-methyltetrahydrofolate) [93]. Concerning cobalamin biostatus, the aggregated biomarker 4cB12, however, can be regarded as the most comprehensive marker of B12 deficiency, as this combined index has been described as more reliable than any single biomarker [51].

Besides the study supplement, other dietary or lifestyle-associated factors may also have potentially influenced Hcy metabolism during the intervention period. The participants were instructed not to change their diet, physical activity, or other lifestyle variables during the intervention. Nevertheless, it cannot be ruled out that mere participation in a nutrition study may have led to increased health awareness causing a plasma Hcy-lowering effect (e.g., lower coffee, protein, and alcohol consumption, methionine overload, less/no smoking and increased physical activity) [80, 94]. However, it is unlikely that these confounding variables occurred exclusively in the supplement group since the plasma Hcy levels remained almost constant in the placebo group. Moreover, according to self-disclosure using questionnaires, the study participants were compliant and no changes in these variables were found.

As described above, reduced kidney function is a major reason for elevated plasma Hcy levels in the elderly, since regeneration of Hcy mainly takes place in the kidney. Reduced renal function may be identified by elevated serum creatinine concentration [95]. However, the mean serum creatinine concentration in our study population was 0.88±0.24 mg/dL and thus, well below the cut-off level for an adverse renal function of >1.4 mg/dL. Therefore, the influence of kidney damage as a confounder was limited.

Nevertheless, we adjusted all relevant statistical models for creatinine level.

Drugs such as metformin or proton pump inhibitors may cause reduced micronutrient availability and may directly interact with Hcy metabolism [37, 96]. We could not identify an association between any medical drug intake and the measured plasma Hcy levels. Medical drugs such as metformin or proton pump inhibitors were only taken by a few subjects (7% of the total population), which is why a systemic influence on the plasma Hcy level can be ruled out. Ham et al. [97] observed that the clinical influence of medication on plasma Hcy levels was smaller than generally assumed.

Strengths and limitations

The strength of the study is the clear and fairly well-characterized cohort, especially considering the applied state-of-the-art biomarkers to evaluate cobalamin and folate biostatus. This study has several potential limitations. We did not analyze genetic mutations in the Hcy-metabolizing enzymes. In detail, isoforms of MTHFR cause reduced folate metabolism and therefore require a higher dose of folate to normalize elevated Hcy levels [98, 99]. However, in our study, the prevalence of low RBC folate levels (<570 nmol/L) was low, so we cannot expect a high influence of this genetic mutation in our population since the prevalence of the heterozygous genotype is approximately 10% anyway [100].

The biostatuses of pyridoxine and riboflavin were not evaluated. Since both vitamins are also involved in Hcy metabolism and are associated with plasma Hcy levels [101, 102], the influence of the pre-interventional supply biostatus of these two vitamins on the plasma Hcy level (e.g., different pyridoxine and riboflavin baseline levels between the verum and placebo groups) cannot be excluded. However, the existing literature indicates that Hcy lowering with pyridoxine is only effective under exceptional circumstances (e.g., methionine overload and severe pyridoxine deficiency) but with a small response [103]. Riboflavin biostatus appears to be a potent modulator in cases of genetic polymorphisms of MTHFR [101, 104]. Thus, there is evidence that the correlation between these vitamins and Hcy levels is considerably low [105, 106]. Consequently, pyridoxine and riboflavin play minor roles in the development of hyperhomocysteinemia in the elderly population. Furthermore, it should be noted that the supplement included both vitamins.

Conclusion

Micronutrient intervention, including several B vitamins, significantly lowered elevated plasma Hcy levels in an elderly population without B vitamin deficiency. Contrary to expectations based on the literature, micronutrient biostatus did not appear to be a determinant of Hcy-lowering effects in this elderly population. At least the studied population of elderly subjects with elevated plasma Hcy levels could benefit from multinutrient supplementation regardless of their nutritional status. The clinical relevance of these findings needs to be investigated in further studies, which should focus on subjects without vitamin B deficiencies. Further investigations are needed to determine the causes of elevated plasma Hcy levels despite the absence of cobalamin and folate deficiencies.

Electronic Supplementary Material

The electronic supplementary material (ESM) is available with the online version of the article at https://doi.org/10.1024/0300-9831/a000777

ESM 1. Linear regression analysis of Hcy levels and levels of serum cobalamin, Holo-TC, and RBC folate at baseline (Table E1).

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Conflict of interest
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Informed Consent Statement
Informed consent was obtained from all subjects involved in the study.

Authorship
Marija Savic-Hartwig and Felix Kerlikowsky contributed equally to this manuscript.

Jan Philipp Schuchardt
Institute of Food Science and Human Nutrition
Leibniz University Hannover
30167 Hannover
Germany
schuchardt@nutrition.uni-hannover.de

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Authors: Felix Kerlikowsky, Karsten Krüger; Andreas Hahn; Jan Philipp Schuchardt

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Effect of a 12-Week Multiple-Micronutrient Supplementation on INFLA Score among Subjects 70 years and older – Results of a Randomised Controlled Study

Felix Kerlikowsky[1]; Karsten Krüger[2]; Andreas Hahn[1],*; Jan Philipp Schuchardt[1],*

[1]Institute of Food Science and Human Nutrition, Leibniz University Hannover, Am Kleinen Felde 30, 30159 Hannover, Germany

[2]Department of Exercise Physiology and Sports Therapy, Institute of Sports Science, Justus-Liebig-University Giessen, Kugelberg 62, 35394 Giessen, Germany

* Both authors contributed equally to this paper

Correspondence: Jan Philipp Schuchardt
Leibniz University Hannover
Institute of Food Science and Human Nutrition
30167 Hannover, Germany
Email: schuchardt@nutrition.uni-hannover.de
Tel.: +49 511 762 2987
Fax: +49 511 762 5729

Abstract

Background: Aging is accompanied by an impaired immune system and chronic low-grade inflammation, while the supply-status of anti-inflammatory micronutrients such as long-chain omega-3 fatty acids, vitamin D, folate and cobalamin is often critical in older people.

Methods: The aim of this randomised, double-blinded, 12-week intervention study with 112 healthy older people (75.5±3.8 years) was to investigate the effect of multiple-micronutrient supplementation in physiological doses (i.e., 400 µg folic acid, 100 µg cobalamin, 50 µg cholecalciferol, 18 mg tocopherol, 100 µg selenium, 1000 mg EPA/DHA) on the INFLA score, a new marker for identifying low-grade inflammation in a holistic approach. The status of the following micronutrients, assumed to be potentially critical, was measured: Omega-3 Index (O3I) for relative EPA+DHA levels of total fatty acids in red blood cells, serum 25-hydroxyvitamin D (25-(OH)D), red blood cell folate (RBC folate), and holotranscobalamin (holoTC).

Results: A significant increase in the nutrient biomarkers in the intervention group compared to the placebo group (all $p<0.001$) was observed. The INFLA-Score slightly decreased in individuals receiving the supplement, whereas the score increased in the placebo group (differences not statistically different). A subgroup analysis revealed a significant decrease in INFLA score in supplement treated subjects ≥80 years, indicating a lower inflammatory state, compared to no change in the placebo group (INFLA score Δt_{12}-t_0: intervention group -4.1±4.5, placebo group 1.3±3.1 with $p=0.022$). Significant associations were found between age and

the decrease in the INFLA score (p=0.010) as well as between the increase in O3I and decrease in INFLA score (p=0.037).

Conclusion: In the present cohort of healthy older people, there was a non-significant reduction in INFLA score with multi-nutrient supplementation in physiological doses. People >80 years of age or with a low basal O3I may benefit from a multinutrient and EPA+DHA supplementation, respectively.

Keywords: INFLA score, inflammaging, Omega-3-Index, Micronutrient supplements, older adults

Trial registration: This study is officially recorded in the German Clinical Trials Register (DRKS00021302, registration date: 23.04.2020).

INTRODUCTION

Aging is accompanied by different physiological changes including alterations in immune function that are characterized by an impaired immune response and a chronic inflammatory status, so-called inflammaging. Chronic low-grade inflammation is characterised by increased inflammatory blood cell counts and the production of various cytokines and chemokines which reinforce inflammatory processes [49,50]. Over- and malnutrition, physical inactivity and smoking, leading to visceral adiposity, can contribute to a persistent pro-inflammatory state [51,52]. Inflammaging may accelerate the aging process or be involved in the development and progression of age-related diseases such as cardiovascular disease [49], neurodegenerative disorders (such as Alzheimer's disease) [53], metabolic syndrome, type 2 diabetes [54] and certain cancers [55].

Diet plays an important role in inflammatory processes [56]. A typical Western diet with a high intake of saturated fats, processed food items, and refined sugars has been linked to the presence of chronic low-grade inflammation [29,57], while a Mediterranean or Nordic diet, rich in antioxidants, minerals, vitamins, and secondary plant-metabolites, can reduce inflammation [58,59]. Individual micronutrients also play a crucial role in the regulation of the immune system. Micronutrients such as arachidonic acid (ARA, C20:4n6) are known as pro-inflammatory, while long-chain omega-3 fatty acids (n3 FA) such as eicosapentaenoic acid (EPA, C20:5n3) and docosahexaenoic acid (DHA, C22:6n3), as well as vitamin D, vitamin C, tocopherols, selenium or zinc are viewed as anti-inflammatory [29,30,56,60,61]. Older people are in particular risk of deficiencies in various micronutrients due to age-related dysfunctions (e.g., reduced mucosal integrity) and an unbalanced diet [14]. In Europe, deficiencies of folate, vitamin D, and cobalamin are highly prevalent, especially among institutionalised [62], but also independently living older people [9,13]. In addition, the supply of long-chain n3 FA is often unfavorable in older people due to low consumption of oily fish and n3 FA-rich plant oils [12].

There is no consensus on which biomarkers are sensitive and specific for the adequate characterisation of low-grade inflammation in human trials [60,61]. The majority of studies that investigated the anti-inflammatory potential of micronutrients, primarily focused on plasmatic inflammatory markers, such as cytokines. However, cytokine assessment may be subject to bias due to short half-lives, assay variability and variations in reagent lots [63–65]. Additionally, there is a lack of age-related cut-offs for cytokine concentrations. As a result, cytokine concentrations alone may not provide a comprehensive assessment of low-grade inflammation.

The INFLA score is a novel marker for identifying low-grade inflammation in a holistic approach, suitable for detecting all stages of the multifactorial immune function. The INFLA score combines CRP concentrations as a “plasmatic biomarker of inflammation”, as well as

the total white blood cell count (WBC), platelet count (PLT) and the granulocyte/lymphocyte ratio (GLR) as “cellular components”. CRP, WBC and PLT are commonly used markers to characterise the inflammatory state or systemic inflammation [66–68]. The GLR detect a rapid and early pro-inflammatory cellular immune response, with an increase in the ratio indicating a shift to a more inflammatory state [69,70]. Higher INFLA scores indicate an increase in low-grade inflammation.

The INFLA score was first applied by Pounis et al. [71] in the MOLI-SANI study to assess the extent of low-grade inflammation in 24,325 Italian adults aged ≥35 years. The authors observed a negative association between the INFLA score and the polyphenol content of the individual diet [71]. In addition, the INFLA score was associated with the Energy Adjusted Dietary Inflammatory Index (E-DII) and the level of processed foods [72]. Moreover, the INFLA score proved to be an independent risk factor for hospitalisation and mortality in an apparently healthy population [73,74]. The question remains whether the anti-inflammatory effect of a multiple micronutrient supplementation can be sensitively assessed using a combination of inflammatory markers such as the INFLA score.

In the present analysis, we aimed to determine and evaluate the effect of MMN on low-grade inflammation using the INFLA score. The overall aim of the study was to assess and improve the status of numerous vitamins, minerals and n3 FA and to investigate the effects of MMN supplementation on metabolism, well-being and immune function in a cohort of healthy older subjects aged ≥70 years.

METHODS

Study design and study procedures

The study was conducted as a single-centre, two-armed, double-blinded, and randomised clinical trial at the Institute of Food Science and Human Nutrition, Leibniz University Hannover, Germany according to the guidelines of the Declaration of Helsinki and registered in the German Clinical Register (DRKS00021302, registration date: 23.04.2020). The study was conducted between March 2021 and November 2021.

Inclusion criteria were age ≥70 years, living home dwelling and independently. The following exclusion criteria were defined: Intake of dietary supplements up to three months before the examination, BMI>35 kg/m^2, severe gastrointestinal or cardiovascular diseases, and intake of immunosuppressants or chronic corticosteroids. The participants provided informed consent before enrolment. 140 subjects were recruited through local press advertisements and announcements in senior network centres and volunteer clubs **(Figure 1)**. Interested subjects were screened for their health status, as well as for the intake of dietary supplements, through a telephone interview.

After controlling for the inclusion and exclusion criteria, the subjects were invited to the Institute for examination. Participants completed a questionnaire regarding their medical history, current medical drug intake (frequency and dosage) and health status, and selected questions on general diet and physical activities. Participants described their movement behavior based on the following classification: “predominantly active” (>2 1/2 hours/week movement with middle intensity or >1 1/4 hours/week with high intensity); “predominantly sedentary” (<2 1/2 hours/week movement with middle intensity or <1 1/4 hours/week with high intensity) or “regular basis movement” (in approximation to 2 1/2 hours/week movement with middle intensity or 1 1/4 hours/week with high intensity).

The examination visits included a measurement of anthropometric data including body weight and height (Seca GmbH & Co. KG, Hamburg, Germany) as well as waist circumferences. Waist circumference (WC) was measured between the lowest rib and the highest hip bone at the narrowest part of the midsection using a tape measure. Blood pressure were performed after a 5 min rest using volume-plethysmography (Boso ABI-system 100; BOSCH & SOHN, Germany) in the left arm. The body composition markers fat mass (FM), lean body mass (BLM), total body water (TBW) and phase angle (PA) were analysed using an 8-point bioelectrical impedance analyser (BIA, mBCA525, Seca Company, Hamburg, Germany). For the measurements, participants were instructed to urinate and remove all jewellery before the examination. Subjects then had to lie down on a stretcher and rest for about 5 minutes to ensure a balanced distribution of body fluids.

All measurements were performed by trained nutritionists of the Institute. The subjects were asked not to change their lifestyle - especially diet and physical activity - during the intervention. At the final examination, the participants completed a second questionnaire regarding changes in medical drug intake, health status, nutrition and movement behavior. Subjects reporting major changes were excluded from the analysis.

Recruitment, screening, study enrolment, and all examinations including blood draw were conducted by stuff members of the institute.

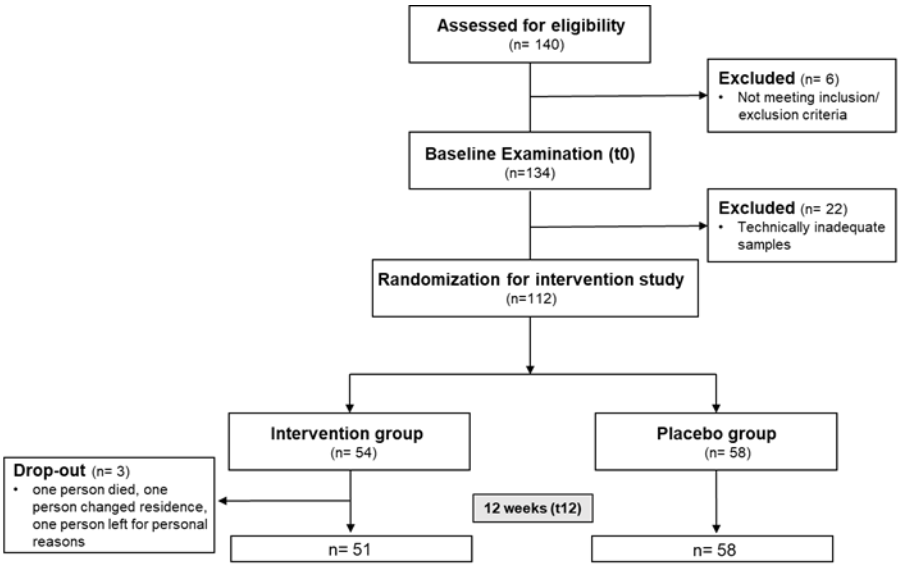

Figure 1: Flow diagram of the study population.

Supplement

Participants in the intervention group were required to take two different micronutrient supplement capsules per day over the duration of 12 weeks. One capsule of the first supplement contained the following micronutrients: 400 µg retinol equivalents (RE), 50 µg cholecalciferol, 18 mg tocopherol (α-TE), 30 µg vitamin K, 200 mg ascorbic acid, 1.65 mg thiamine, 2.1 mg riboflavin, 16 mg niacin equivalent (NE), 2.1 mg pyridoxine, 400 µg folic acid, 100 µg cobalamin, 50 µg biotin, 6.0 mg pantothenic acid, 10 mg zinc, 100 µg selenium, 40 µg chromium, 50 µg molybdenum, 100 µg iodine. One capsule of the second supplement contained 500 mg EPA and 500 mg DHA. Two placebo preparations were administered. The placebo group had to consume one capsule consisting of 500 mg maltodextrin and one capsule with 1.0 g evening primrose oil. The verum and placebo capsules had the same size and appearance.

Blood sampling

Blood samples were collected between 08:00 and 11:00 a.m. at baseline (t_0) and final examination (t_{12}) after overnight fasting (minimum 12 h fasting period). When possible, participants were invited to t_{12} at the same time of the day as the t_0 examination. Blood samples were obtained by venipuncture of the arm vein using Safety-Multifly® needles (Sarstedt, Nümbrecht, Germany) into serum, EDTA, or S-Monovettes® for tHcy (Sarstedt). Samples were stored at approximately 5 °C and transferred to the laboratory on the same day.

Biochemical analyses

Plasma concentration of **CRP** were determined using a human Magnetic Luminex Assay (Bio-Techne, Abingdon, Oxon, UK) and a Magpix Luminex instrument (Luminex Corp, Austin,

Texas, US) according to the manufacturer's instruction. Serum **25-(OH)D** was measured in duplicate at SYNLAB MVZ (Leinfelden, Germany) using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS, Recipe, Munich, Germany). Serum **holoTC** was determined using electrochemiluminescence immunoassay (ECLIA) on cobas® test systems (Roche Diagnostics GmbH, Mannheim, Germany) as previously described [75,76]. **RBC folate** was analysed using ECLIA on Immulite 2000 analyser series (Diagnostic Products Corporation, Los Angeles, USA). Concentrations of vitamins are reported in nmol/L, in case of holoTC and in pmol/L. CRP concentrations in mg/dL. All numbers were rounded to three significant digits. **Complete blood count** analysis (including a differential white blood cell count) was performed using fluorescence flow cytometry (Fluorocell PLT, XN-9000, Norderstedt, Germany). GLR values were calculated by dividing the sum of the absolute neutrophil, basophil and eosinophil counts by the absolute lymphocyte count. CRP, 25-(OH)D, holoTC, RBC folate, and blood count Biochemical analyses were determined in an accredited and certified laboratoriesy (LADR Laborärztliche Arbeitsgemeinschaft, Hannover, Germany; SYNLAB MVZ, Leinfelden, Germany)

The **O3I,** defined as the EPA+DHA content of RBC as a percentage of total identified fatty acids, is viewed as the preferred biomarker for evaluating the long-term n3 PUFA status in clinical practice and research [77,78]. In the present study, the O3I was analysed in an accredited and certified laboratory (OmegaQuant Analytics, Sioux Falls, SD, USA) using dry blood spots (DBS [79] and gas chromatography) [80]. Briefly, frozen EDTA blood tubes were thawed and spun for 15 min at 2000 rpm for 15 min to isolate the RBC fraction. After removal of the plasma and buffy coat, 100 uL of packed RBC were mixed with 100 µL of an "erythrosolve" solution (3 mg EDTA per mL of normal saline) to increase the adhesion of the RBCs on the filter cards. 50 µL of the dissolved RBCs were spotted onto OmegaQuant oxidant-pre-treated DBS filter cards. The filter cards were sent to OmegaQuant Analytics for fatty acid analysis.

Calculation of the INFLA score

The INFLA score was calculated as shown in **figure 2**.

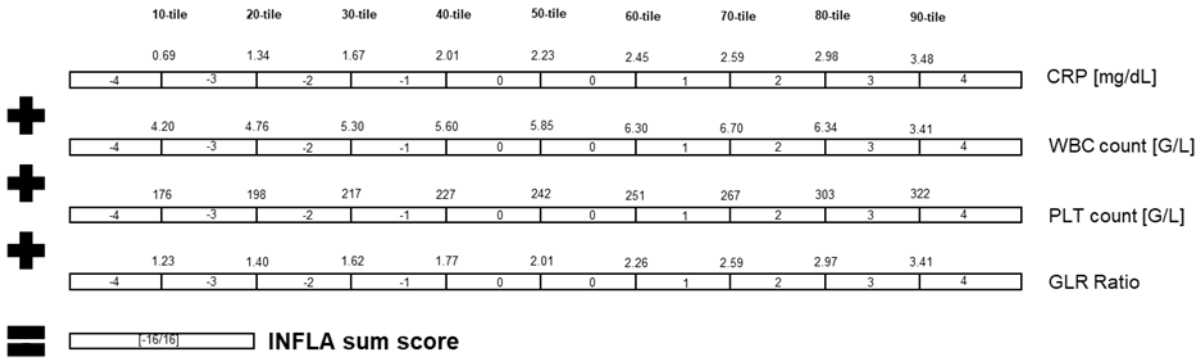

Figure 2: Calculation of the INFLA score.

Legend: The INFLA score was obtained by generating 10-tiles of plasmatic CRP and cellular biomarkers: white blood cell (WBC) count, platelet count (PLT) and granulocyte/lymphocyte ratio (GLR). For all biomarkers, the four highest 10-tiles were given positive scores ranging from 1 to 4. The four lowest 10-tiles of all biomarkers were given negative scores ranging from -1 to -4. Any 10-tile between the four highest and lowest 10-tiles was scored as zero. Finally, the INFLA score was calculated as the sum of the four biomarkers for each subject and examination day.

Subgroup analysation

To detect differences in the change in INFLA score, we stratified the overall cohort into three age classes (Table S1) resulting in subgroups of subjects aged 70 to 74 years (n=59), 75 to 79 years (n=37), and 80 years and older (n=16).

Sample size calculation and statistical analysis

Sample size was calculated using an expected drop-out rate of 10%, a significance level of 5%, and a power of 80%. To detect differences in the two-sided t-test between the verum and placebo groups with a Cohen's effect size of 0.5, a case number of 60 subjects per group (n=120 in total) was obtained.

Continuous variables are shown as mean±standard deviation (SD), while qualitative variables are presented either as absolute or relative frequencies, or only in relative figures. The Shapiro-Wilk test was used to test for normal distribution. In addition, quantile-quantile plots were created for visual inspection. Intention-to-treat analysis was performed to avoid potential bias owing to the exclusion of subjects. The Student's t-test was used to compare variables in the case of normally distributed data. The chi-squared test was used to determine the distribution of nominal variables between the intervention and placebo groups. Finally, to assess the intervention effect of the INFLA score and the micronutrient biomarkers, a general linear model (GLM) with repeated measures was used with time (t_0, t_{12}) as the within-subject factor and intervention as the between-subject factor. The statistical model was further controlled for covariates: sex, BMI and age. Linear regression models were used to detect associations between baseline characteristics of the subjects and the intervention effect and between variation in micronutrient biomarkers and the intervention effect, adjusted for sex, BMI and age. For all analyses, statistical significance was set at p-levels <0.05. Statistical analyses were performed using SPSS software (IBM SPSS Statistics28.0; Chicago, IL, USA).

RESULTS

Characteristics of the participants

The majority (74%) of the 112 older subjects, who finished the study, were women (**Table 1**). Overall, the subjects had a mean age of 75.5±3.9 years. Mean BMI (25.6±4.7 kg/m^2) and waist

circumference (female: 90.7±12.3 cm, male: 98.3±8.9 cm) were within the physiological range for older people compared with the general German population aged 70-75 years (mean BMI: 27.0 kg/m^2 [81]). In addition, the cohort was highly educated (54% high school education), current non-smokers (97%), and physically active (92% regularly or mostly active) compared to the general German population aged 65-75 years (30% with a high school education, 88% non-smokers, 64% regularly or mostly active [81,82]). At baseline examination, there were no differences in demographic, anthropometric and lifestyle markers between the intervention and placebo groups (Table 1).

Table 1: Baseline characteristics of the study population.

	Total n=112	**Intervention n=54**	**Placebo n=58**	**p-value**
	Mean±SD	**Mean±SD**	**Mean±SD**	
Age [y]	75.5±3.9	75.4±4.3	75.7±3.5	0.736 [a]
70-74, n [%]	59 (52)	32 (59)	27 (47)	
75-79, n [%]	37 (33)	15 (28)	22 (38)	0.944 [b]
≥ 80, n [%]	16 (15)	7 (13)	9 (15)	
Sex, male, n [%]	30 (26)	14 (26)	13 (22)	0.415 [a]
BMI [kg/m^2]	25.6±4.7	25.1±4.0	26.0±5.3	0.287 [a]
Female	25.5±5.2	25.1±4.5	25.9±5.8	0.497 [a]
Male	25.6±2.9	24.9±2.2	26.4±3.3	0.154 [a]
Waist circumference [cm]	92.5±11.9	92.1±11.0	92.9±12.9	0.734 [a]
Female	90.7±12.3	90.4±11.3	90.9±13.1	0.857 [a]
Male	98.3±8.9	97.0±8.5	99.8±9.5	0.426 [a]
Blood pressure [mmHg]				
Systolic	143±15.6	142±15.6	144±15.7	0.492 [a]
Diastolic	84.3±11.3	84.1±10.0	84.5±12.4	0.852 [a]
Education level				
Low education, n [%]	18 (16)	12 (20)	6 (12)	
Middle education, n [%]	34 (30)	15 (30)	18 (31)	0.341 [b]
High education, n [%]	60 (54)	27 (50)	34 (57)	
Smoking status				
Current smoker, n [%]	4 (3)	0 (0)	4 (7)	
Previous smoker, n [%]	50 (45)	22 (41)	28 (48)	0.178 [b]
Never smoke, n [%]	58 (52)	32 (59)	26 (45)	
Diet				
Omnivor, n [%]	94 (85)	47 (85)	48 (83)	0.545 [b]
Vegetarian, n [%]	18 (15)	7 (15)	10 (17)	
Physical activity				
Predominantly active, n [%]	28 (25)	13 (24)	15 (26)	
Predominantly sedentary, n [%]	9 (8)	5 (9)	4 (7)	0.846 [b]
Regular basis movement, n [%]	75 (67)	36 (67)	39 (67)	

[a] students t-test between intervention group and placebo group, [b] chi-squared test.

Effect of supplementation on selected nutrient status markers and the INFLA score

At the baseline examination, no differences in any of the nutrient status markers between the intervention and placebo group were observed (**Table 2**). As expected, after 12 weeks of supplementation, we noticed a significant increase in O3I, RBC folate, holoTC and 25-(OH)D

(all p<0.001), while all markers were unchanged in the placebo group. At t_{12}, O3I, RBC folate, holoTC and 25-(OH)D were significantly higher compared to the placebo group.

Likewise, there was no difference in the INFLA score between the intervention and placebo group at baseline. After 12 weeks of intervention, the INFLA score slightly decreased in individuals receiving the MMN supplement, whereas the INFLA score increased in the placebo group. The differences were not statistically different. After adjustment for sex, BMI and age, the interaction remains non-significant, noting that within the intervention group there was a slight reduction within the INFLA score.

Table 2 Mean levels of INFLA score and nutrient status markers at t_0 and t_{12}.

						p-value (ANCOVA)	
Variables	**n**	**Intervention group**	**n**	**Placebo group**	**Group**	**Time**	**Interaction**
		Mean±SD		Mean±SD			
INFLA score [-16/16]							
t_0	54	-0.04±5.85	58	0.36±5.69	0.715†		
t_{12}	51	-0.53±6.25	58	0.76±6.07	0.278†		
Δ t_{12}- t_0	51	-0.17±4.39	58	0.40±4.09		0.594 [a]	0.372 [b]
O3I [% of total fatty acids]							
t_0	54	6.0±1.3	58	6.4±1.4	0.127†		
t_{12}	51	7.9±1.2	58	6.3±1.4	**<0.001†**		
Δ t_{12}- t_0	51	1.7±0.9	58	-0.1±0.8		**<0.001** [a]	**<0.001** [b]
RBC folate [nmol/L]							
t_0	54	805±221	58	831±246	0.587†		
t_{12}	51	1140±226	58	870±266	**<0.001†**		
Δ t_{12}- t_0	51	343±226	58	38.68±18.2		**0.003** [a]	**<0.001** [b]
HoloTC [pmol/L]							
t_0	54	84.5±31.0	58	91.5±35.0	0.573†		
t_{12}	51	103±32.8	58	86.4±34.6	**0.004†**		
Δ t_{12}- t_0	51	16.8±19.8	58	-5.0±16.7		**0.001** [a]	**<0.001** [b]
25-(OH)D [nmol/L]							
t_0	54	81.2±23.6	58	87.3±27.6	0.209†		
t_{12}	51	112±24.2	58	81.9±29.2	**<0.001†**		
Δ t_{12}- t_0	51	30.2±21.5	58	-5.1±15.0		**<0.001** [a]	**<0.001** [b]

Data are mean±SD. P-values represent: †students t-test between intervention group and placebo group and analysed using generalized linear models with time (pre/post) and intervention as fixed factors: [a] time interaction, [b] time*intervention*sex*BMI*Age interaction.

Association of age with changes in INFLA score

Age at baseline was significantly associated with a change in the INFLA score in subjects of the intervention group (**Figure 3a**). Moreover, in a subgroup of subjects ≥80 years, the INFLA score significantly decreased in the intervention group, while the INFLA score was unchanged

in subjects ≥80 years in the placebo group (intervention group Δ t_{12}-t_0: -4.1±4.6, placebo group: 1.3±3.1, Table S1). Consequently, the changes in INFLA score differed significantly (p=0.022) between the intervention and placebo group (**Figure 3b**).

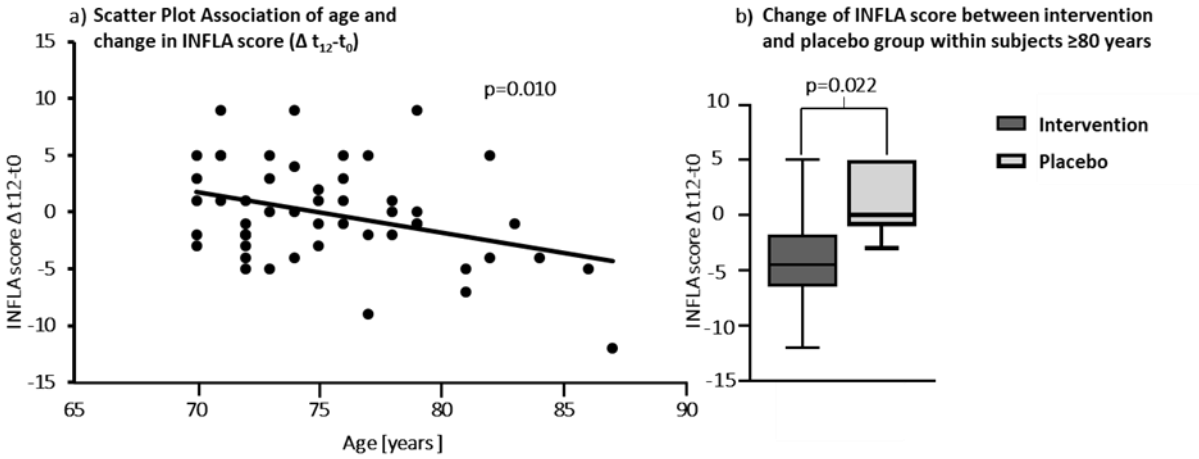

Figure: 3 Influence of age on change in INFLA score

Legend: a) Association between age and change in INFLA score (Δ t_{12}-t0), adjusted for sex and BMI, p=0.010, R^2=0.095, Regression coefficient=-0.325, b) Change of INFLA score between intervention and placebo group within subjects ≥80 years.

Association of nutrient status marker with changes in INFLA score

The observed increase in O3I was significantly associated with a decrease in INFLA score (**Figure 4 a)**, while the decrease in relative ARA level in RBC was significantly associated with a decrease in INFLA score. **(Figure 4 b)**. No associations were found between the change in INFLA score and changes in other nutrient status markers.

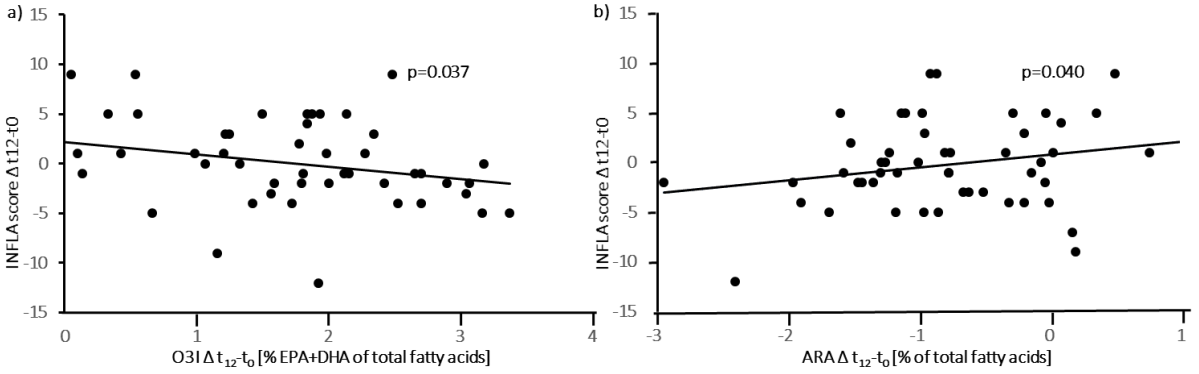

Figure 4: Association between change in INFLA score and change a) Omega-3 Index (O3I) and b) relative ARA level in RBC.

Legend: a) Linear regression model adjusted for sex, BMI and age, p=0.037, R^2=0.039, Regression coefficient=-1.549 b) Linear regression model adjusted for sex, BMI and age, p=0.040, R^2=0.047, Regression coefficient=1.680.

DISCUSSION:

The present study aimed to assess the effect of MMN supplementation on low-grade inflammation using the INFLA score in a cohort of healthy older subjects aged ≥70.

We found a slight, non-significant reduction in the INFLA score at 12 weeks. In subjects aged 80 years and older, the reduction in INLFA score was significant. Furthermore, the increase in O3I was significantly associated with a decrease in INFLA score. No associations were found between serum concentration of 25-(OH)D, holo-TC and RBC folate with changes in INFLA score.

Irrespective of the anti-inflammatory effects of individual micronutrients [30], several randomised controlled trials (RCTs) investigating the effect of MMN on inflammatory markers in older people predominantly failed to show significant effects [83–85]. These studies varied in duration, age (mainly in the 60s), disease setting (healthy independently living older vs. patients with chronic heart failure), MMN composition (mainly without n3 FA), and focused only on pro-inflammatory cytokines as markers of inflammatory status. In this study, we used the INFLA score as a novel and comprehensive marker of inflammation that has not been used in RCTs.

The observed change in the INFLA score cannot be evaluated because no cut-off values have been defined up to now. The basal mean+SD INFLA score of 0.17±5.75 in our study is in a comparable range to that of the MOLI-SANI study (-0.20±5.90) [73]. While a slight increase (n.s.) in the INFLA score was observed in the placebo group, a non-significant decrease in the INFLA score was found in the intervention group. The reasons for the weak effect of the MMN intervention on the INFLA score could be manifold: insufficient duration of the intervention period, composition and dosage of the micronutrients in the MMN supplement used, a large age range in the study cohort, an inhomogeneous study cohort in terms of inflammatory status (resulting in high inter-individual variability of INFLA score), and finally the very good health condition of our study cohort (characterised by an average low BMI, blood pressure and waist circumference, low prevalence of current smokers) resulting in a low average basal INFLA score. Finally, the good supply status of anti-inflammatory micronutrients may be particularly relevant for weak effects.

In this study, age also seems to influence the anti-inflammatory effect of the MMN intervention. We observed a significant association between the decrease in INFLA score and age. In the subgroup of people over 80 years of age the decrease in INFLA score in response to the MMN intervention was significant. A possible reason for the age-related effect may be a shift of low-grade inflammation from older to oldest old [86,87]. As expected, basal INFLA scores tended to be higher in the group aged > 80 years (Table S1), which is in line with a higher BMI, body

fat and waist circumference compared to younger subjects (Table S2). A body composition characterised by high levels of adipose tissue responds above a non-specific level with immune activation and secretion of pro-inflammatory mediators [88]. Moreover, the accumulation of senescent cells with age leads to detrimental metabolic effects, defined as the senescence-associated secretory phenotype (SASP). The SASP mediates the activation and recruitment of both adaptive and innate immune cells and the secretion of pro-inflammatory cytokines [89].

As the MMN supplement used is a multi-nutrient preparation, it is unclear which nutrient affects the immunomodulatory and metabolic pathways involved in inflammatory processes. We measured endogenous status markers for micronutrients that are often deficient in the older and in the general population, and that have recently been recommended for supplementation in this dosage and composition to support a well-functioning immune system [90,91].

We found that the increase in O3I was significantly associated with a decrease in INFLA score. The potential of long-chain n3 FA, and in particular EPA and DHA, to resolve low-grade inflammation has been shown in previous studies [29,60]. However, there are few comparable studies of n3 FA supplementation on low-grade inflammation in healthy older people over 70 years of age.

In a previous study with healthy older adults (mean age: 64 years), we observed a significant reductions in Tumor necrosis factor (TNF_a) and IL-6, but not in CRP, in response to high-dose n3 FA supplementation (1320 mg EPA + 880 mg DHA) over 26 weeks [92]. Basal O3I in n3 FA treatment group was unexpectedly high at 8.0%, and therefore in a range that is suggested to be desirable for cardiovascular and brain function [93]. In the present cohort, the basal O3I was considerably lower at 6.2+1.3%, but comparable to the older German population [12]. After n3 FA supplementation, the O3I in our study reached 7.9+1.2%, which is to be expected with the given n3 FA dose, but below the 8% cut-off. However, it is unclear whether a certain O3I cut-off needs to be exceeded in order to achieve anti-inflammatory effects. It is possible that anti-inflammatory effects only occur above a yet unknown amount of n3 FA in cell membranes and that the O3I achieved in our study was not sufficient to induce anti-inflammatory effects.

A study by Kiecolt-Glaser et al. [94] used a comparable dose of EPA/DHA (1250 mg per day) compared to our study and observed a significant reduction in TNF_a and IL-6 in 138 healthy middle-aged adults (51±7.8 years). However, the EPA:DHA ratio in the n3 FA supplement they used was 7:1, whereas the EPA:DHA ratio in our n3 FA supplement was 1:1. EPA and DHA have different metabolic effects and possibly unique effects on inflammation [95]. However, the anti-inflammatory potential of EPA vs. DHA is controversial [96].

Besides the optimal EPA:DHA ratio, there is also a debate about the dose required for anti-inflammatory effects. Recent reviews [60,97] suggest that 2000 mg EPA+DHA per day is required to achieve anti-inflammatory effects, although this amount depends on the basal O3I levels. Studies investigating the anti-inflammatory effects of different doses of EPA+DHA are rare and provide inconsistent results [97–99]. Bouwens et al. [98] found no significant changes in plasma CRP concentrations in 111 older subjects (>65 years) after supplementation of a low-dose (0.4 g EPA+DHA per day) or high-dose (1.8 g EPA+DHA per day). Similarly, Sukulas-Ray et al. [97] observed no difference in cytokine concentrations after supplementation with a high dose (3.4 g EPA+DHA per day) and low dose (0.85 g EPA+DHA per day) in 28 healthy middle-aged adults (mean age: 44.3 years). In contrast, Flock et al. [99] observed a modest lowering effect on TNF_a concentrations after supplementation with a high dose (1.8 g EPA+DHA per day) to 125 healthy young adults (mean age: 26.1 years) for 5 months, while no effects were observed with lower doses of n3 FA. There were no effects on IL-6 or CRP concentrations. We can only speculate whether higher doses of n3 FA would have greater effects on the INFLA score in our study.

Nevertheless, the incorporation of n3 FA into cell membranes in exchange for n6 FA is dose-dependent, along with several other factors [time-dependency, age, genetics, diet], and potential anti-inflammatory effects are based on increased amounts of n3 FA in cell membranes [100]. As a result of a higher amount of n3 FA compared to n6 FA, cell membranes become more fluid, which in turn modulates protein function and pro-inflammatory signaling events [101]. In addition, after hydrolysis from the cell membranes, free EPA and DHA can inhibit inflammatory processes by converting via multiple hydroxylations into bioactive metabolites such as resolvins, protectins and maresins, collectively known as specialised pro-resolving mediators (SPMs) [102]. Besides, n3 FA reduces the formation of pro-inflammatory ARA-derived eicosanoids [60]. Furthermore, free EPA and DHA inhibit leukocyte chemotaxis, adhesion molecule expression and leukocyte-endothelial adhesive interactions, and reduces the activation of nuclear factor-kB (nf-kB), a transcription factor that induces the expression of several pro-inflammatory genes, such as cytokines.

Besides n3 FA, the MMN supplement contained other micronutrients that have been reported to possess anti-inflammatory effects (e.g., vitamin D, zinc, selenium, vitamin C) [29]. Although we observed a significant increase in serum 25-(OH)D concentration, this increase was not associated with changes in INFLA score. Many of the older subjects in the cohort were physically highly active. As the study was conducted during the summer period and in the middle of the COVID-19 pandemic, when activities were only allowed outdoors, the subjects were exposed to UVB radiation. Taken together, this may explain the overall good vitamin D status at baseline. Additional markers of nutrient status in this study, such as RBC folate or holoTC, were not significantly associated with changes in INFLA scores. This may be due to

the relatively good supply status with folate and cobalamin in the cohort. The prevalence of folate and cobalamin deficiency, based on the status markers RBC folate or holoTC, was low (data not shown). Nevertheless, even if we do not see any associations between status markers and changes in the INFLA score, an effect of these micronutrients from the MMN supplement on inflammatory processes cannot be ruled out.

Strengths and limitations:

The strength of the study was the straight forward design using the INFLA score as an inflammatory marker of low-grade inflammation, including plasmatic and cellular components of the immune system. To our knowledge, the MMN supplement used was the first to combine a vitamin, mineral and n3 FA supplement for the older population at physiological doses. Moreover, we used state-of-the-art biomarkers to assess long-chain n3 FA, vitamin D, cobalamin and folate status.

However, our study had a number of potential limitations. First, the small sample size was very small, especially within the population aged 80 years and older. However, this was the first pilot intervention study to investigate the effect of MMN supplementation on INFLA score in the older population. Vitamin C and selenium status was not assessed. As both micronutrients have been described to be involved in immune function, we cannot exclude associations between INFLA score and these micronutrients. Finally, as the study cohort consisted mainly of highly educated, active and health-conscious individuals, the results cannot be extrapolated to the average community-dwelling older population in Germany, which does not have the same health-consciousness and physical activity.

CONCLUSION:

12 weeks of MMN supplementation resulted in a non-significant reduction in INFLA score in a healthy older population. Further research should focus on older people aged 80 years and above, where we observed a greater reduction in the INFLA score and thus a potential anti-inflammatory effect. As the decrease in the INFLA score was dependent on the increase in O3I, future studies should investigate the effect of different n3 FA doses and EPA:DHA ratios in older subjects with low basal O3I. The classification of the INFLA score requires further research.

Declarations

Ethics approval and consent to participate

The ethic committee of the medical chamber of Lower Saxony (Hannover, Germany) approved all procedures. Written informed consent was obtained from all participants prior to their enrolment.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interest

The authors declare no conflict of interests.

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Authors´ contributions

FK: Data acquisition, evaluation and curation, writing-original draft preparation; JPS, AH: Conceptualization and study design, methodology, writing, reviewing and editing, supervision; All authors have read and agreed to the submitted version of the manuscript.

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5. General discussion

The aim of this thesis was to investigate the nutrient status of healthy, active and independent living older people over 70 years of age who were not taking micronutrient supplements, and to assess whether this otherwise healthy population could benefit from taking a multi-micronutrient supplement for 12 weeks. The data were generated in a single-centre, two-arm, double-blind, randomised clinical trial at the Institute of Food Science and Human Nutrition, Leibniz University Hannover, Germany. The results of the data analysis are presented in the scientific publications in **Chapter 2-4**.

The following is intended to classify the reported results of this thesis in the existing scientific literature.

5.1. Vitamin D status in healthy and active older people (Paper I):

Vitamin D is recognised as a critical nutrient in the general population. The prevalence of deficiency is estimated to be between 16-80% in the European area [14]. Endogenous synthesis is the most important factor influencing vitamin D status. The capacity of endogenous synthesis may be significantly reduced with age. Therefore, the prevalence of vitamin D insufficiency is thought to be particularly high in older people. [92]. This has been shown in previous studies, mainly assessing vitamin D status in older people living in nursing homes or with pre-existing metabolic diseases, who are also restricted in their daily physical activity and therefore exposure to sunlight (Table 1). There is a paucity of cross-sectional observational studies investigating vitamin D status in healthy, independent, physically active older people who do not take supplements.

Main results compared with previous studies:

- The prevalence of sufficient serum concentration of 25-(OH)D (50 nmol/L and above) were high with 93%.
- However, 7% of the older cohort did not achieve sufficient vitamin D status and in detail 2 subjects were serve deficient with serum concentrations of 25-(OH)D below 25 nmol/L.
- Serum concentrations of 25-(OH)D did not differ between female and male older people (female: 85.9±26.8 nmol/L, male: 83.1±23.8 nmol/L, total: 85.1±26.0 nmol/L)
- Age at baseline was not significant associated with measured serum concentrations of 25-(OH)D. However, lowest mean serum concentration of 25-(OH)D were measured within the subgroup of people 80 years and older.

The intake of 25-(OH)D, as determined by 3-day food records, was 4.1±5.0 µg per day. However, vitamin D intake was not significantly associated with serum concentration

- of 25-(OH)D in a fully adjusted model (adjusted for age, sex, energy intake, body weight, BMI, WC and creatinine).

Only a few studies examinated the vitamin D status in unsupplemented older cohort have also found a surprisingly good vitamin D status. In an older population in Japan, 54.4% of subjects achieved a serum concentration of 25-(OH)D above 50 nmol/L and 17.4% above 75 nmol/L [214]. An observation by Grootswagers et al. [97] in the Netherlands found a similarly high serum 25-(OH)D concentration of 79.9 nmol/L in an older cohort. In August in Germany, Klenk et al. [82] also found a high mean serum 25-(OH)D concentration of 77.6 nmol/L and a low prevalence of vitamin D insufficiency of 16.1%. The comparable studies were similar in terms of mean age, BMI, gender distribution, and seasonality of testing. Therefore, this study confirms that adequate vitamin D status can be achieved even in an older population during months of high UV irradiation. However, the mean serum concentration of 25-(OH)D in this cohort was quite high at 85.1±26.0 nmol/L, and most existing data to date have shown significantly lower serum concentrations of 25-(OH)D and higher prevalence of vitamin D insufficiency in both the general population and the old population [14]. The following factors influencing vitamin D status can be discussed as possible explanations for the almost adequate vitamin D status in this study.

Factors that influence the vitamin D status

Exposure to UVB radiation is the main determinant of serum 25-(OH)D concentration from April to September [82]. However, in the older population, factors such as immobility, social isolation, depression, and living situations with limited outdoor activities can lead to inadequate UV exposure even in the summer months [215]. That's why previous studies of older people living in nursing homes have shown even worse vitamin D status than this study. Calculations from the USA and the Netherlands showed that skin synthesis of 25-(OH)D was only 12.5 µg-15 µg per day in the months of June or July, resulting in almost insufficient vitamin D status if only skin synthesis is considered [216]. However, this cohort was characterised by a high level of physical activity, travel and exercise, mostly outdoors, due to the COVID 19 pandemic in 2021. In addition, it should be taken into account that older people, unlike younger people, are not affected by occupational duties and may be more likely to be active and outdoors during the period of maximum UVB radiation during the day, leading to higher synthesis of 25-(OH)D via the skin.

Secondly, **individual body fat mass and BMI** significant affect the vitamin D status [217, 218]. Fat tissue acts as a depot for fat-soluble vitamin D and deprives it of its bioavailability in the body. In this study as well as in the comparative studies reported relatively high vitamin D

concentrations, the collective was characterised by an normal range of BMI (20-30 in subjects aged 70 years and older).

Age-related changes also affect kidney function. **Impaired kidney function** negatively affects vitamin D status [219]. On the one hand, through reduced 1_{α}-hydroxylase activity, but also through increased losses of 25-(OH)D via the urine. The cohort in this study was characterised by physiological creatinine concentrations (women: 70.4±8.8 µmol/l and men 88.0±17.7 µmol/l), which reflect normal renal function [220] (laboratory specific reference ranges: women: 44.9-83.6 µmol/l; men: 59.0-103 µmol/l) and may be one of the reasons for the adequate serum concentrations of 25-(OH)D in this cohort.

The dietary intake of vitamin D in this study (98% of all participants did not meet the DRI for vitamin D of 20 µg per day in the absence of sunlight exposure) was comparable to the general German population, where 94% of men and 97% of women did not meet the DRI. However, dietary intake of vitamin D typically meets up to 10-20% of the vitamin D requirement [221]. A cross-sectional evaluation of the B-PROOF (B-vitamins for the Prevention of Osteoporotic Fractures) in 2857 adults aged 65 years and older assessed a mean vitamin D intake of 4.9 µg per day [222]. The intake of vitamin D was significant associated with the measured serum concentration of 25-(OH)D in winter and in summer time. The authors explain that in summer and autumn, each µg of vitamin D intake increases the serum concentration of 25-(OH)D by 1 nmol/L. However, vitamin D intake in this cohort was low with 4.1±5.0 µg per day, which leads to the conclusion that dietary intake did not have a significant effect on vitamin D status in this study.

Another very interesting finding from the cross-sectional analysis of the B-PROOF study was that **genetic variability** explained 28% of the variation in serum concentrations of 25-(OH)D [222]. Thus, genetic make-up had a similar strong influence on vitamin D status in older people as UVB exposure. However, the influence of different genotypes on vitamin D status could not be determined in this study.

Non-individual specific determinants that influence reported vitamin D status are based on the chosen **laboratory analytic method** for vitamin D assessment [223]. In this study, we chose the LC-MS/MS method, which is described as more specific and reflects more valid data than the comparable CLIA method, which may underestimate existing serum concentrations of 25-(OH)D. In addition in this study, serum concentrations of 25-(OH)D were measured twice, with similar results. However, in previous research, both methods of analysis are commonly used in those reporting a high prevalence of vitamin D deficiency in older people and in those with similar results to this study.

5.2. Folate and cobalamin status in healthy and active older people (Paper I):

These are the first data on folate- and cobalamin status in healthy, active and independent living older people in Germany who were not taking micronutrient supplements using validated long-term functional markers (holoTC and RBC folate) as well as metabolic markers (hcy and MMA) and an aggregated biomarker (4cB12).

Main results compared with previous studies:

- 70% of older women and 62% of older men did not meet the DRI for folic acid equivalents.
- Dietary intake of folic acid was significantly associated with RBC folate concentration in a fully adjusted model (adjusted for age, sex, energy intake, body weight, BMI, WC and creatinine)
- The prevalence of deficient RBC folate concentrations was low in both female (10%) and male (12%) subjects.
- Age had no influence on the measured RBC concentrations in this cohort.
- Dietary intake of cobalamin undercut the DRI for cobalamin in 55% of the total population.
- There was no association between measured holoTC concentrations and cobalamin intake.
- The prevalence of deficient holoTC concentrations in this study was low in female (8%) but high in males (22%).
- Age was not significantly associated with the concentration of holoTC.
- Using 4cB12 as an aggregated marker of cobalamin status 87% in total achieved a calculated 4cB12 count classified as adequate cobalamin status.
- 63.1% of the older subjects had elevated hcy concentrations and 37.1% elevated MMA concentrations.

Factors that influence folate and cobalamin status

Comparison with previous research is limited because previous assessments focused mainly on serum biomarkers, which are strongly influenced by acute dietary intake [122,126]. In observations where **valid long-term markers** are assessed, the study design of comparable studies is limited by the fact that the cohort was partially supplemented or the studies were conducted in countries with folic acid fortification. In addition, the prevalence of deficiency was often not assessed by well-established and frequently used cut-offs. Particularly in the case of RBC folate, a wide range of **assay-specific cut-offs** have been established, leading to different classifications of adequate RBC folate concentrations. An observation by Wolters et

al. [136] found a prevalence of deficient RBC folate concentrations in 2.3% of older female subjects living independently using 320 nmol/L as a cut-off, whereas Gonzales-Gross et al. [137] used a cut-off of 400 nmol/L and reported a prevalence of deficiency of 18.1% in older people living in nursing homes. In this study, we used an assay-specific cut-off for RBC folate deficiency of 570 nmol/L, which is much higher than comparable cut-offs used. However, considering the high mean RBC folate concentrations achieved in this study (831±244 nmol/L), the applied cut-off targeted subjects who clearly had low RBC concentrations. In addition, subjects with RBC folate concentrations below 570 nmol/L had significantly lower serum concentrations of folate and significantly higher plasma concentrations of hcy compared with subjects who are not deficient in RBC folate, supporting the assumption of a deficient folate status (data not shown).

Furthermore, the studies compared differ in the **living conditions** of the people studied. It can be assumed that in our cohort the independent living status leads to a more self-determined and healthy food choice, characterised by a high intake of fruit and vegetables. Fruit and vegetable intake has already been shown to be associated with RBC folate concentration, and this intake was higher in this study than in the German population aged 65-80 years [224].

In contrast to vitamin D, an adequate status of folate cannot be synthesised endogenously. It should be borne in mind that folate is a heat-labile vitamin and intensive heating processes reduce the **bioavailability of folate from dietary sources** [81]. It can be assumed that older subjects in good general health choose their food independently and are able to consume several dishes without intensive heating processes. This may lead to a selection of foods rich in folate and a higher bioavailability of the foods consumed. In addition to several limitations in the assessment of dietary intake data, the assumption of a high adsorption rate of folate in healthy elderly may be supported by the significant association between folate intake data and RBC concentrations in this study.

In addition, several **medications** have been described that interact with folate and may reduce its bioavailability [225]. There are conflicting data between medical drugs such as metformin [226], proton pump inhibitors [227] and cytostatics [228], which may predict higher folate depletion. However, in this cohort of healthy older people, the use of medications that may interact with folate is extremely low or absent.

Genetic MTHFR polymorphism such as C677T are linked to reduced bioavailability of folate. Prevalence in European ethnic groups ranges from 10% to 32% [229]. MTHFR genotype was not assessed in this study. The MTHFR 677TT genotype is associated with reduced RBC folate concentrations [230]. We can only speculate that the prevalence of MTHFR

polymorphism in this cohort was quite low, based on the relatively high mean RBC folate concentrations and the low prevalence of RBC folate deficiency.

Previous observations show that **serum creatinine concentration** is negatively associated with serum folate concentration [231]. However, this association cannot be extrapolated to RBC folate because erythrocytes are normally not excreted in the urine. Nevertheless, in this healthy cohort of older people, renal function as assessed by serum creatinine concentrations was close to physiological, which can only support the high prevalence of adequate folate status.

Folate and cobalamin interact in their metabolic function. This also applies to their circulating concentrations in the blood, which are dynamically and positively linked [231].

In older population, **holoTC** has been reported to be a sensitive marker for the assessment of cobalamin status, even among those with the impaired renal function [121]. In this study, the prevalence of to low holoTC concentrations was low with 12% of the total cohort. This is much lower than in previous studies: Herrmann et al. [138] reported a prevalence of holoTC deficiency of 28% in an older German cohort living in the community and in institutions, whereas the prevalence of deficiency among older people living in nursing homes was 39% [137]. Both comparable studies used a lower cut-off (35 pmol/L and 45 pmol/L) compared to our study with 50 pmol/L. However, the difference found compared to previous research underlines the good health status of this sub cohort of healthy, active and independently living older people.

Nevertheless, our results showed gender difference with a significantly higher prevalence of deficiency in older men than in older women. The observed difference between men and women is consistent with findings from the National Health and Nutrition Examination Survey (NHANES) of 1,770 older subjects in the USA, where men had a significantly higher risk of cobalamin deficiency than women [232, 233]. Reasonable for **sex difference** in cobalamin status in this cohort may be that men consumed slightly less vitamin B_{12} when considering energy-adjusted intake (men: 3.4±5.7 µg per day, women: 3.9±1.8 µg per day compared the DRI of 4.0 µg per day).

However, the main cause of inadequate cobalamin status in older people is **malabsorption of food-bound cobalamin** [81]. Gastric diseases such as H. pylori infection and atrophic gastritis are mainly causal for the malabsorption of food-bound cobalamin in the older population [226]. However, the current study included a healthy population. Subjects with severe gastrointestinal disease were not included. Therefore, we can only speculate that the prevalence of H. pylori infection and atrophic gastritis was quite low in this cohort. However, H. pylori infection may be a causal factor for the sex difference observed in this study. In previous studies, the

prevalence of H. pylori infection was higher in men than in women, which could explain the lower HoloTC concentration in older men [234]. In addition, single-nucleotide polymorphism (SNP) are **genetic factors** which may explain sex difference in cobalamin status [233]. Specifically, the gene fucosyltransferase 2 may be responsible for lower cobalamin absorption in men. However, this topic is not well studied. In our cohort, we did not assess genetic variations. Furthermore, a **plant-based diet, smoking, alcohol consumption, low educational level and chronic use of proton pump inhibitors** are all associated with low cobalamin status [233]. In our data, older men did not show a higher prevalence of these factors than older women.

5.3. Effects of multi micronutrient supplementation on nutrient status and metabolic parameters in healthy older people (Paper II)

MMN supplements refers to any supplement containing 3 or more vitamins and minerals, unless herbs, hormones or drugs [235]. The primary goal of the use is to counteract an unbalanced diet and resulting micronutrient gaps, defined as an intake below the DRI as given by professional nutrition societies. Secondly, the question remains whether the resulting improvement in micronutrient status has additional benefits for metabolic health. Hcy is a metabolic parameter that has been scientific studied for decades, particularly in the older population [153]. One of the aims of this thesis was to improve the status of critical micronutrients in older people and to investigate their impact on a number of health-related biomarkers including the hcy concentrations.

Main results compared with previous studies:

- MMN supplement significantly improved the concentrations of RBC folate and holoTC compared to no changes in the placebo group.
- The prevalence of deficient RBC folate concentration decreases to almost zero after 12 weeks of intervention.
- The prevalence of deficient holoTC concentration decreases from 12.1% to 1.6% within the group of MMN supplementation.
- In the entire cohort, the mean plasma hcy concentration at baseline was 18.2 µmol/L. The prevalence of an elevated hcy concentration (≥15 µmol/L) was 63.2%.
- Before the intervention plasma concentration of hcy were significant associated with age, holoTC concentration but not with concentration of RBC folate.
- After 12 weeks of intervention with the MMN supplement, the prevalence of elevated hcy concentrations decreased to 35.5% with a mean reduction of 4.0±5.7 µmol/L, while there were no changes in the placebo group.

- The decrease in hcy concentration within the intervention group were significant associated with baseline hcy concentration.
- The decrease in hcy concentration within the intervention group occurred regardless of baseline folate and cobalamin status.

Effectiveness of MMN supplementation in reducing elevated hcy concentrations

MMN supplements containing different **compositions and doses of B vitamins** (folic acid with or without cobalamin and pyridoxine) are already shown to be an effective strategy to reduce elevated hcy concentrations [153]. The reduction observed in previous RCTs using physiological doses of folic acid ranged from 9.8 to 48.6% and was slightly improved by additional supplementation of cobalamin, whereas additional supplementation of pyridoxine may only be effective in reducing elevated hcy concentrations in methionine overload and severe pyridoxine deficiency [128]. Based on the almost sufficient folate and cobalamin status in this cohort, the observed 23% reduction in hcy concentrations underlines the efficacy of MMN supplements for metabolic health in older people, regardless of B vitamin status. However, we have not analysed the biostatus of pyridoxine and riboflavin. We can only provide information on the status of cobalamin and folate.

The mechanism of action is scientifically well understood and based on the need of folic acid, cobalamin and pyridoxine within the mehtionine-homocystein metabolism (**Figure 3**). However, it is not well understood scientifically whether additional folic acid and cobalamin supplementation beyond adequate levels is helpful in reducing elevated concentrations of hcy. Previous studies on this topic are limited by the lack of valid biomarkers to assess baseline cobalamin and folate status. In addition the comparison with previous studies is limited by the fact that previous studies mostly included people with a history of cardiovascular events or one or more risk factors for CVD. Nevertheless, a few comparable studies have arrived similar conclusions based on related vitamins in normal ranges [236, 237].

Despite the initial B vitamin status, two decades of intensive scientific research into whether B vitamin supplementation can reduce hcy concentrations, and whether this is secondary to the prevention of cardiovascular events have failed to demonstrate a reduced risk of CVD [153]. It is important to bear in mind that, in addition to the status of individual micronutrients, several different factors may be causal for elevated hcy concentrations and also for the risk of CVD. In the older population, **reduced kidney function** and **lifestyle factors** such as smoking and alcohol consumption as well as a lack of physical activity are associated with elevated hcy concentrations, with **men tending to have higher levels than women** [238–240].

At the very least, the supplement used in this study differs from previous studies investigating the effect of B vitamins on hcy concentrations by adding antioxidants (ascorbic acid,

tocopherol, zinc and selenium) and n3 FAs. Antioxidants may reduce oxidative stress in older people who otherwise have beneficial effects on other metabolic pathways and n3 FA may induce enzymes involved in hcy metabolism via modulation of gene expression [241]. However, to date there are only cross-sectional data showing an association between antioxidant intake and hcy concentrations, which does not reflect a causal relationship [242, 243].

5.4. Effects of multi micronutrient supplementation on inflammatory biomarker in healthy older people (Paper III)

Inflammageing is one of the key manifestations of impaired immune function in the ageing process, as described earlier in this thesis. Due to the complexity of the immune system, systemic meta-analyses have failed to classify inflammaeging by a single biomarker [244]. In addition, assessment of plasma concentrations of pro- and anti-inflammatory cytokines has failed to define inflammageing due to a lack of standardised ranges indicating normal concentrations in older people. [244, 245]. In this thesis we assessed the inflammatory status of healthy older people using the INFLA score. The INFLA score is a novel marker that combines CRP concentrations as a "plasmatic biomarker of inflammation", as well WBC count, PLT and GLR as "cellular components in a holistic approach and therefore suitable for detecting all stages of multifactorial immune function" [77]. The calculated INFLA score ranges from -16 to 16 points, with a lower score indicating a lower degree of low-grade inflammation.

Main results compared with previous studies:

- As shown in Paper I, this study cohort was predominantly well supplied with the nutrients vitamin D, folate and cobalamin at baseline.
- In contrast, before the intervention, 88% of the older subjects did not achieve a desirable O3I >8%.
- After 12 weeks of MMN supplementation, O3I increased by 1.7%±0.9%. The prevalence of subjects not achieving a desirable O3I was 55% in the intervention group and still high at 85% in the placebo group.
- The INFLA score slightly decreased in individuals receiving the MMN supplement, whereas the INFLA score slightly increased in the placebo group.
- Age at baseline was significantly associated with the assessed decrease in the INFLA score.
- Subgroup analysis showed a significant reduction in INFLA score in subjects aged 80 years and older.

- Positive effects on the INFLA score within the intervention group were significantly associated with the increases in the O3I but not associated with changes in serum concentrations of 25-(OH)D, RBC folate or holoTC.

Effectiveness of MMN supplementation to combat inflammation in older age

Inflammation is known to usually underline (and drive) poor metabolic health [246]. However, elevated pro-inflammatory cytokines have also been observed in healthy, successfully ageing older people [247]. The calculated INFLA score in this study (0.17±5.75) cannot be definitively classified as pro- or anti-inflammatory, as no cut-off values have yet been defined. However, the mean and SD of the INFLA score in this study was comparable to the INFLA score within the MOLI-SANI study (-0.20±5.90), where the INFLA score was first described [248]. However, the population studied in the MOLI-SANI examination was much younger (53±10.9 years) than this cohort of healthy and active older people aged 70 years and above. Nevertheless, the calculated INFLA score was almost similar, which may reflect a **low baseline level of inflammation** in our cohort. In conclusion nutritional support by a MMN supplement may be more effective when inflammatory processes are more pronounced. For instance, in hospitalised patients with various types of cancer, where CRP levels are 10 to 100 times higher at baseline, nutritional support significantly reduces elevated CRP levels and reduces mortality over 30 days [249].

In addition, the **very good health status and low prevalence of micronutrient deficiencies** in this study cohort may explain the lack of significant effects. However, at baseline, 88% of the older subjects in this cohort did not achieve a desirable O3I of >8%. This means that, in contrast to vitamin D, cobalamin and folate status, the status of n3 FA in this cohort was almost critical. Starting from a critical status of n3 FA may explain why we only found significant associations between increase in O3I and decrease in the INFLA score and not between increase in vitamin D, cobalamin and folate status and decrease in the INFLA score. Previous clinical trials investigating the potential of long-chain n3 FA, particularly EPA and DHA, to resolve low-grade inflammation have varied in dose, duration of supplementation, EPA:DHA ratio, and study characterisation [250–252]. Specifically, in a study by Witte et al. [253], where reductions in TNF_a and IL-6, but not in CRP were observed, the O3I achieved at the end of the intervention was almost desirable at 9.7±2.9%, whereas in our study the O3I achieved after the intervention with the MMN supplement was still below 8% on average at 7.9%±1.2%. However, for the risk of fatal CVD, only an O3I >8% is considered desirable. We can only speculate whether a specific content of EPA and DHA in erythrocyte membranes is necessary to achieve anti-inflammatory effects, and whether a higher dose or duration of treatment

leading to an improvement in intervention subjects of more than 8% would have significant effects on the INFLA score.

Age has also been shown to influence the n3 FA profile [254]. With increasing age, higher levels of EPA and DHA have been observed in erythrocytes, possibly due to a higher incorporation efficiency of these FA into cell membranes, although this is still under discussion [255] . Specifically, the difference was found between young and old adults and not between old and very old. However, in this study we noticed a significant reduction in the INFLA score only in subjects aged 80 years and older. In addition, subjects aged 80 years and older achieved a slightly higher, but almost above 8% (8.5%±1.5%) O3I at the end of the intervention compared to younger subjects (7.8%±1.4%). As mentioned above, it may be that the anti-inflammatory effects of n3 FA only occur above an as yet unknown level of n3 FA in cell membranes, which may be comparable to the 8% threshold suggested to be desirable for cardiovascular and brain function [181]. However, subjects aged 80 years and older also had a shift in the INFLA score, reflecting a higher initial state of inflammation. The age-related increase in inflammation, is consistent with higher BMI, body fat and waist circumference compared to younger subjects in this cohort. Both, older age and a body composition characterised by high levels of adipose tissue respond above a non-specific level with immune activation and secretion of pro-inflammatory mediators [256, 257]. Due to the small sample size in this study, we cannot clarify which factors explain most of the variance in the decrease in INFLA score in treated subjects aged 80 years and older.

Previous work has shown that the INFLA score is associated with the Dietary Inflammatory Index (DII) and the level of processed foods [258]. Therefore, in addition to adequate micronutrient intake, the **quality of the background or basis diet** plays a key role in inflammatory processes [259]. A typically western diet which is high in arachidonic acid, sweetened drinks, refined sugars or highly processed foods may counteract the potential beneficial effects of several micronutrients in resolving inflammatory effects [30, 260]. The opposite has been shown in studies examining the effects of the DASH diet, the Nordic diet and especially the Mediterranean diet [260–262]. All of these diets are high in fruit and vegetables, vegetable oils, oily fish, nuts and whole grains, and provide sufficient antioxidants, minerals, vitamins and phytochemicals to be responsible for the anti-inflammatory effects [263, 264]. We can only speculate whether the quality of the background diet in our cohort significantly influenced the effect of MMN supplementation on the INFLA score. However, vegetable and fish intake in this cohort was higher than in the general German population aged 65-80 years [90]. Nevertheless, linear regression analysis showed no association between the amount of vegetables, fruit or fish consumed and the initial INFLA score.

5.5. Strengths and limitations

The strength of this study was its straightforward design with well-characterised subjects. In addition, we used state-of-the-art analytical parameters and methods to measure vitamin D, folate and cobalamin status and O3I. Furthermore, we used a MMN supplement in physiological doses, to investigate effects that can be translated into practice-relevant recommendations. Previous studies investigating the effect of MMN supplements have mostly failed to add n3 FA to the MMN supplement. In this study, we combined a medium-high dose of n3 FA with a MMN supplement that is commonly used in daily practice. In addition, this study was the first RCT to investigate the effects of a MMN, including n3 FA, on plasmatic and cellular markers of inflammation, aggregated as the INFLA score.

The human study conducted for this thesis has a number of potential limitations:

Firstly, the sample size was very small, particularly for the interpretation of subgroup analyses. However, this was the first pilot intervention study to investigate the effect of MMN supplementation on metabolic health and inflammation in an unsupplemented older population with a healthy and active lifestyle.

Secondly, we did not assess the status of all supplemented micronutrients, such as vitamin C, selenium, riboflavin, pyridoxine and thiamine, which would improve the overall assessment of the nutrient status of this cohort and may also interact with the changes in metabolic and inflammatory parameters observed in this study. However, a strength of this study was that it only assessed micronutrient status using valid long-term markers, which are not currently available for micronutrients such as vitamin C, selenium, riboflavin, pyridoxine and thiamine.

Thirdly, to better assess the effect of MMN supplementation on metabolic health and immune function, information on oxidative status and cell senescence (T-cell phenotyping) would be very helpful to underpin the reported findings.

Finally, as the study cohort consisted mainly of highly educated, active and health-conscious individuals, the results cannot be extrapolated to the average community-dwelling older population in Germany, which does not have the same health-consciousness and physical activity.

6. General conclusion and perspectives

This thesis contributes to a better understanding of critical micronutrients in the older population. It has been shown that older people living independently, with a high level of education, physical activity and health awareness, are predominantly well supplied with vitamin D, folate and cobalamin, which are otherwise critical in the general population and particularly

in older people living in nursing homes. Despite, a healthy and active lifestyle a not to be underestimated proportion achieved a deficient status of the assessed micronutrients and consequently benefit from taking MMN supplement. In particular, the intake of n3 FA assessed by the O3I was almost not desirable, which is similar to findings in the general German population. The O3I improved significantly after 12 weeks of taking middle high doses of n3 FA as part of the MMN supplement. However, the dose and duration used were not sufficient for all older people to reach a desirable O3I level above 8%.

In addition, this thesis demonstrates that older people with normal renal function have a high prevalence of elevated hcy concentrations. Regardless, of folate and cobalamin status, older people benefit from a 12-week MMN supplementation through a significant reduction in elevated hcy concentrations. This is in contrast to the cardinal principle of nutrition that only people with an initial deficiency will benefit from supplementation.

A 12-week MMN supplementation leads to a non-significant reduction in the INFLA score overall and a significant reduction in subjects aged 80 years and above. This demonstrate that MMN supplementation can modulate inflammatory biomarkers in healthy, active and predominantly well-supplied older people. The effect depended on age and increase in O3I. Still, the biological significance of this reduction remains questionable, science initial low-grade inflammation was not very pronounced. Beyond the significant association between the decrease in INFLA score and the increase in O3I, it remains unclear which individual micronutrients within the MMN supplement are most responsible for the anti-inflammatory effects or, as previously postulated [265], whether a specific combination is required to combat inflammation in the old age. This study provides further strong evidence for the anti-inflammatory effects of n3 FAs, which is consistent with findings from studies using single micronutrient supplements.

The findings on vitamin D, folate and cobalamin status in this thesis should be consolidated in further observations focusing explicitly on subjects of advanced age, 80 years and older. In addition, it should be further investigated whether hcy concentrations occur in individuals with adequate folate and cobalamin status and whether these individuals would benefit from a reduction in elevated hcy concentrations by MMN supplementation towards CVD and cognitive decline. Further studies are needed to validate the INFLA score as a tool to measure low-grade inflammation and whether the INFLA score can be modified by dietary strategies, particularly in people aged 80 years and older.

7. References

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Scientific publications derived from this thesis

Original research papers (peer-review)

I. **Kerlikowsky F**, Schuchardt JP, Hahn A (2023): Sufficient Status of Vitamin D, Cobalamin and Folate in Healthy and Active German Home-Dwelling People Over 70 Years of Age. *BMC Geriatrics. 2023 Oct.*

II. Savic-Hartwig M***, Kerlikowsky F****, van de Flierdt E, Hahn A, Schuchardt JP (2023): A micronutrient supplement modulates homocysteine levels regardless of vitamin B biostatus in elderly subjects. *Int J Vitam Nutr Res. 2023 Jan.*

III. **Kerlikowsky F**, Schuchardt JP, Krüger K, Hahn A (2023): Effect of a 12-Week Multiple-Micronutrient Supplementation on INFLA Score among Elderly Subjects – results of a randomized controlled study (submitted *to BMC Geriatrics. 2023 Aug.*)

* Authors contributed equally to this work

Co-authored publications

IV. Lenz J, Tintle N, **Kerlikowsky F**,Badrasawi M, Zahdeh R, Qasrawi R, Hahn A, Schuchardt JP (2023): Assessment of the vitamin D status and its determinants in young healthy students from Palestine. J Nutr Sci. 2023 Mar.

V. Staufenbiel I, Adam K, Hahn A, **Kerlikowsky F**, Flohr M, Schlueter N, Vach K (2023): Influence of Nutrition and Physical Activity on Local and Systemic Inflammatory Signs in Experimentally Induced Gingivitis. *Nutrients 2023 Jul.*

VI. **Kerlikowsky F**, Müller M, Greupner T, Amend L, Strowig T, Hahn A (2023) Distinct Microbial Taxa Are Associated with LDL-Cholesterol Reduction after 12 Weeks of Lactobacillus plantarum Intake in Mild Hypercholesterolemia: Results of a Randomized Controlled Study. *Probiotics Antimircob Proteins 2023 Oct.*

Conference contributions

Posters

Kerlikowsky F, Greupner T, Müller M, Espadaler-Mazo J, Hahn A (2021): Probiotic formulation influcences blood cholesterol levels: A randomized, controlled trial during the Covid-19 pandemic, 43. European Society for Clinical Nutrition and Metabolism (ESPEN) Congress (Virtual), 9.9.-14.9.2021

Kerlikowsky F, van de Flierdt E, Hahn A, Schuchardt JP (2023): A micronutrient supplement modulates elevated homocysteine concentrations regardless of vitamin B biostatus in elderly

subjects, 60. Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung, Rheinische Friedrich-Wilhelms-Universität Bonn, 15.3.-17.3.2023.

Kerlikowsky F, Greupner T, Hahn A, (2023): Ein Methodenvergleich von LC-MS/MS und CLIA zur Messung von 25-(OH)D im Serum, Regionalverband Nord, Lebensmittelchemische Gesellschaft, Fachgruppe in der GDCh, Hannover, Arbeitstagung, 20.3-21.3.2023.

Kerlikowsky F, Krüger K, Schuchardt JP, Hahn A (2023): Folate, Vitamin B_{12} and Vitamin D status in healthy and active home-dwelling people over 70 years, International Vitamin Conference Copenhagen, 20.9-22.9.

Kerlikowsky F, Krüger K, Hahn A, Schuchardt JP (2023): Effect of a 12-Week Multiple-Micronutrient Supplementation on INFLA Score among Subjects 70 years and older – Results of a Randomised Controlled Study, International Vitamin Conference Copenhagen, 20.9-22.9.

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Appendix Curriculum vitae

Persönliche Daten	
Name	Felix Kerlikowsky
Geburtsdatum und -ort	12.08.1991 in Anklam
Berufserfahrung	
seit 04/2020	Wissenschaftlicher Mitarbeiter, Institut für Lebensmittelwissenschaft und Humanernährung, Leibniz Universität Hannover
Hochschulausbildung	
seit 04/2020	Promotionsstudium am Institut für Lebensmittelwissenschaft und Humanernährung, Leibniz Universität Hannover
10/2014-03/2020	Studium der Veterinärmedizin (med.vet.): Stiftung Tierärztliche Hochschule Hannover (TiHo); Abschluss Staatsexamen
10/2011-09/2014	Studium der Ernährungswissenschaften, Martin-Luther-Universität (MLU) Halle (Saale); Abschluss Bachelor of Science (B.Sc.)
Schulabschluss	
06/2010	Allgemeine Hochschulreife, Lilienthal-Gymnasium, Anklam