Nutritional strategies to improve muscle quality during ageing

Pol Grootswagers

Propositions

1. Nutritional strategies can improve physical performance in older adults even in the absence of concurrent exercise (This thesis)
2. Handgrip strength assessment should be replaced by measuring knee extension strength for the monitoring of changes in muscle strength in older adults (This thesis)
3. Having equal parental leave is the most important step towards closing the gender wage gap (*Andersen, 2018*)
4. The pirate website 'Sci-Hub' that provides access to scientific literature to everyone, should be applauded for accelerating open science more than legal initiatives do
5. Documentaries that claim to be 'scientific' should undergo peer-review
6. Society should celebrate, rather than fear, the progressive replacement of jobs by AI technology
7. Older adults make better life-coaches than 30-year-olds

Propositions belonging to the thesis, entitled

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Chapter 1

Introduction

Increased life expectancy

Human life expectancy is increasing. Advances in medicine, hygiene and safety make people survive longer than ever before. Life expectancy in the Netherlands is projected to reach 86 years in the year 2040 [1]. Such an increase in lifespan is not only a great achievement of humankind, but the additional years of life also create new opportunities, such as starting further education or even a new career. The extent to which additional years can be used to pursue such opportunities is, for a large part, dependent on health and independence [2]. Older adults report a desire to stay socially active, mentally active and physically active during ageing [3]. To be able to succeed in these domains, older adults should maintain an adequate level of physical performance [4]. However, physical performance in older adults is often compromised [5].

The ageing muscle

During ageing, muscle mass decreases. After the age of 30, people lose on average three to eight per cent of muscle mass every decade [6, 7]. Such loss implicates that if a person has 35 kg of muscle mass at the age of 30, this person might end up with between 23 kg and 30 kg muscle mass remaining at the age of 80. One of the reasons for this age-related loss in muscle mass is a decreased exposure to anabolic stimuli later in life, such as physical activity [8], protein intake [9] and anabolic hormones [10]. Besides, the anabolic response to these stimuli is blunted in older people, a phenomenon that is termed *anabolic resistance* [11].

Underneath this visible muscle mass decline, a change that is less obvious but arguably as important is happening: a declining muscle strength. During ageing, muscle strength is decreasing at a much faster rate than muscle mass does. The estimated loss in muscle strength is between 10 and 20% per decade up to the age of 70 [12], and between 20% and 30% in the decades thereafter [13]. The loss in muscle strength is considered a more important risk factor for functional decline than the loss in muscle mass [14]. Muscle strength might, therefore, be more relevant than muscle mass for quality of life of older adults. This notion is indeed confirmed by the work of Balogun et al. [15] They compared community-dwelling older adults in the lowest 20% of handgrip strength or lower-limb muscle strength with those with normal strength levels. They found a significant, and clinically meaningful, lower health-related quality of life over 10 years of follow-up for those with low strength. The associations between low appendicular mean mass and health-related quality of

life were weaker, indicating that muscle strength is a stronger determinant of quality of life than muscle mass [15].

The growing understanding of the more important role for muscle strength than for muscle mass in predicting functional decline and quality of life is reflected in the development of the sarcopenia guidelines. Where the initial definitions of sarcopenia were only based on muscle mass, the 2010 revised guidelines added muscle function to the definition [16]. The most recent guidelines of 2019 have even put muscle strength assessment in the first place of sarcopenia diagnosis [17]. **Figure 1** shows the consensus-based algorithm to find cases for sarcopenia diagnosis, with the critical position of muscle strength assessment in this algorithm highlighted in yellow. For the evaluation of muscle strength the working group advises the assessment of handgrip strength, by calibrated dynamometry under standardised conditions [18], or the chair rise test, a test in which participants have to stand up from a chair five times as quickly as possible, without using their arms [19].

The working group acknowledge the limitation of handgrip strength measurements in those with hand disabilities caused by, for instance, arthritis or stroke. For these people, they advise the measurement of isometric knee extension torque [17]. Recently, further criticism was shown towards the use of handgrip measurements to assess muscle strength. Tieland et al. showed that handgrip strength failed to reflect considerable increases in muscle strength [20]. Other studies have shown that, compared to handgrip strength, knee extension strength is a better predictor of functional performance [21] and is more strongly associated with health characteristics [22]. Isometric knee extension torque or force assessment might prove to be a more valid alternative, but more research on the validity of knee extension strength assessment methods that are suitable for clinical practice is still needed [23].

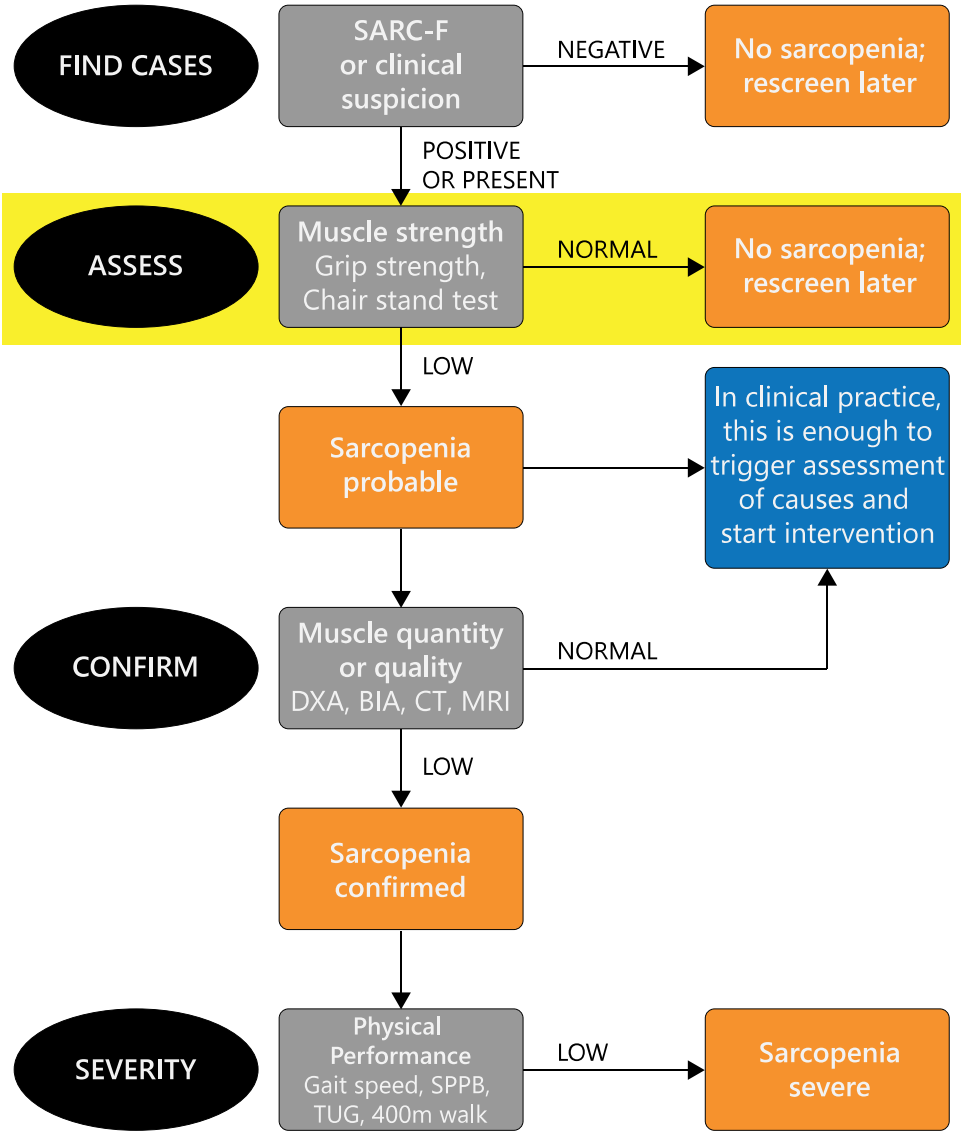

Figure 1. Case-finding algorithm for sarcopenia diagnosis, as recommended by the European Working Group on Sarcopenia in Older People 2. Adapted from [17]. Notice that this algorithm advises the measurement of muscle quality via imaging techniques to assess the amount of fat infiltration, or to quantify muscle area, volume or mass to calculate relative strength.

Muscle quality

The observation that muscle strength is lost at a higher rate than mass reveals that muscle function is not solely determined by muscle mass. The functioning of a muscle beyond its mass can be called muscle quality [24]. The pragmatic definition of muscle quality is function per mass and can be calculated as muscle strength or power per unit of mass, volume or cross-sectional area [24]. A more sophisticated way of evaluating muscle quality is to examine the determinants of relative muscle strength themselves [25]. Four important determinants of muscle quality are type II muscle fibre atrophy, mitochondrial functioning, fat infiltration, and neuromuscular activation (**Figure 2**). The bottom layer of **Figure 2** shows the lifestyle factors that can improve determinants of muscle quality. These evidence from randomised controlled trials with exercise, nutrition and bio-actives in older adults is summarised in **Table 1-3**.

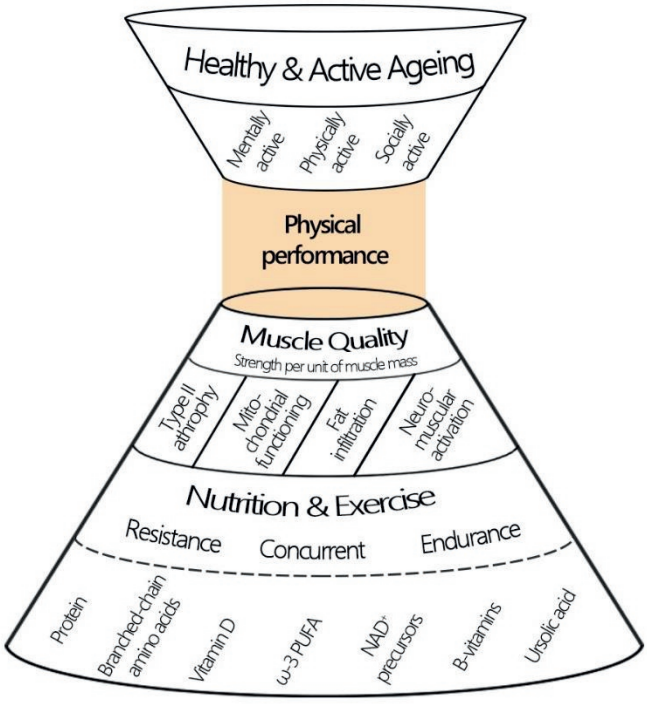

Figure 2. Schematic representation of the possible influence of nutrition and exercise on healthy ageing via muscle quality-related mediators of physical performance.

Type II atrophy

Specific type II muscle fibre atrophy is an important determinant of age-related decreases in muscle mass and quality. In muscle tissue, we distinguish two fibre types: type I and type II [26]. Type I fibres have a higher abundance of mitochondria and oxidative enzymes [27], and are more abundant in the skeletal muscle tissue of endurance athletes [28]. The type II fibres (including type IIa, IIb and IIx) have a higher glycolytic enzyme activity than type I fibres have [29], but they are more prone to fatigue [30]. Type II fibres are more abundant in the skeletal muscle tissue of sprinters and weight lifters [28]. During ageing, specific atrophy of type II muscle fibre size occurs. Research shows that the size of type II muscle fibres is 10 to 40% smaller in older compared to young adults [31-34]. Interestingly, one study showed that the difference in type II fibre size fully explained between-group differences in quadriceps size [31].

The cross-sectional area of type II muscle fibres correlates not only with muscle mass, but also with leg strength [35]. Targeting age-related type II fibre atrophy is, therefore, a potential strategy to improve muscle quality. Studies show promising results on improving type II fibre size via resistance exercise [36-48] (**Table 1**). It should be noted that there is an equal amount of RCTs that fail to show improvements in type II fibre size [49-61], which could be due to different training regimens or duration, sample characteristics, or low sample size. Other types of exercise, such as endurance exercise and electrostimulation, do not seem to improve type II fibre size (**Table 1**).

Protein could theoretically augment resistance exercise-induced improvements in type II fibre size by allowing muscle protein synthesis after resistance exercise [62]. However, only one trial [63] of a total of nine showed positive effects of protein supplementation on type II fibre size in older adults (**Table 2**). Moreover, trials with leucine [64], vitamin D [65, 66], antioxidants [67], creatine [53, 55] and chromium picolinate [37, 52] failed to improve type II fibre size in older adults, suggesting that this determinant of muscle quality should be targeted predominantly via resistance exercise. Resistance exercise could be combined with supplementation of anabolic nutrients, to obtain postulated additional improvements in type II fibre size [68], but this strategy needs further investigation in older adults.

Mitochondrial functioning

Apart from focussing on improving muscle strength via increasing the size of type II fibres, another promising strategy to improve muscle quality is by improving mitochondrial functioning in skeletal muscle tissue. Due to the specific atrophy of type II fibers, muscles of older adults have a higher proportion of type I fibres, which have a greater abundance of mitochondria [27]. Yet, the number of mitochondria and the mitochondrial oxidative capacity are reduced in older individuals [69]. Sarcopenic individuals show an even further reduced mitochondrial oxidative capacity [70]. In these individuals, the biosynthesis of nicotinamide adenine dinucleotide (NAD^+) is also repressed [70]. Studies show that lower mitochondrial functioning relates to lower physical performance in older adults [71, 72].

Nutrition might stimulate mitochondrial functioning via multiple targets [73]. For instance, dietary intake of NAD^+-precursors, such as vitamin B3 and tryptophan, can lead to increased levels of NAD^+ [74, 75]. Moreover, certain bio-actives, such as ursolic acid, seem to directly improve mitochondrial biogenesis in mice models [76]. In older adults, the few trials that have tested the effects of different nutrients, or bio-actives, on mitochondrial functioning, all found positive results [77-80] (see **Table 2** and **Table 3**). Regarding muscle quality, targeting the mitochondria might be the approach that is most adaptive to subtle changes in the environments such as obtained by nutritional interventions. Apart from interventions with nutrients or bio-actives, endurance training and high-intensity interval training seem effective in improving mitochondrial functioning too (see **Table 1**).

Fat infiltration

A third determinant of muscle quality is the infiltration of fat tissue in the muscle. The cross-sectional images of quadriceps muscle tissue presented in **Figure 3**, show clear intramuscular adipose tissue (IMAT) in the 66-year-old person (B) compared to the 24-year-old person (A) [81]. While IMAT does contribute to quadriceps volume and to muscle mass, it negatively affects muscle strength [82, 83] or muscle endurance [82]. Fat infiltration is, therefore, an illustrative cause of a loss in muscle quality, as it directly decreases the muscle strength per unit of muscle size. Fat infiltration may be an essential risk factor for functional decline [84]. The first studies in older adults show that exercise programs can decrease IMAT [85] or prevent IMAT accumulation [86]. That IMAT accumulation in the muscle is preventable by lifestyle is also visible in **Figure 3c**. This quadriceps belongs to a 66-year-old male, with a

high level of physical activity. The quadriceps of this person shows amounts of IMAT that are more comparable to those of the 24-year-old male (A), than to those of the 66-year- old sedentary male (B). This suggests that physical activity might diminish the effects of ageing on IMAT accumulation. Yet, exercise interventions in older adults have yielded conflicting results (**Table 1**).

Aside from physical activity, nutrition might play a role in IMAT accumulation too. Increased IMAT is observed in older adults at risk of malnutrition [87], with diabetes [88], and it is related to increased insulin resistance [89, 90]. These findings highlight the metabolic aspects of IMAT and suggest a role for nutritional interventions to prevent or decrease IMAT build up. The first dietary interventions aimed at lowering IMAT in older adults have been finished recently, with positive results for supplementing whey protein and vitamin D [91, 92].

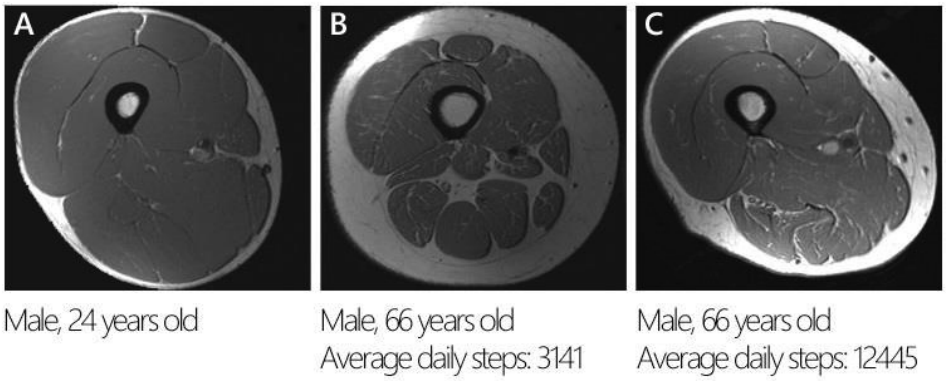

Figure 3. Cross-sectional image of a quadriceps of (A) a 24-year old male, (B) a 66-year old male and (C) a 66-year old male with a high level of physical activity. Adapted from McLeod et al 2016 [81]

Neuromuscular activation

In some cases, the loss in strength output of a muscle is not caused by morphological changes within the muscle tissue, but by decreased neuromuscular activation and the loss of motor units [93]. Typically during ageing, we observe a loss in the voluntary activation [94]. Decreased voluntary activation is related to decreased physical functioning in older adults [95-97]. Nutrients like vitamin D [98] and homocysteine-lowering nutrients vitamin B6, B12 and folate [99, 100] are postulated to improve neuromuscular activation. However, the direct effect of these nutrients on neuromuscular outcomes are rarely measured [101] (**Table 2 and 3**). Nutrients tested in randomised controlled trials that do show to improve neuromuscular activation in older adults are creatine [102], milk fat globule membrane [103] and N-3 fatty acids [104]. The exercise regimens that are most effective in improving neuromuscular activation are resistance training and power training (**Table 1**).

Thesis Outline

An active lifestyle seems to prevent losses in muscle quality to some extent, and exercise programs can improve markers of muscle quality. Not many intervention trials have aimed to improve muscle quality via nutrition, despite the fact that metabolic aspects play a role in muscle quality, and many nutrients act on pathways that are related to physiological determinants of muscle quality. Together, this gives reasons to pursue the identification of nutritional strategies to improve muscle quality. This thesis aims to identify novel nutritional strategies to improve muscle quality and physical performance in older adults.

In **chapter 2**, the reliability and validity of hand-held dynamometry are investigated. Hand-held dynamometry forms a practical alternative to Biodex for leg strength assessment in older adults, which is important in muscle quality assessment. **Chapter 3** assesses the effectiveness of 4-week nutrition and exercise intervention on muscle mass, muscle strength and muscle quality. In **chapter 4**, the effects of a novel oral nutritional supplement on muscle mass and muscle functioning in older adults with (or at risk of) undernutrition. **Chapter 5** explores the role of B-vitamins in relation to physical functioning in healthy older adults. Copula graphical modelling, which is a statistical method that is new in the field of nutrition, is used in **chapter 6** to identify new nutritional targets to improve physical functioning in older adults with different levels of health. Finally, in **chapter 7**, the results of this thesis are discussed, and directions for future research and implementation are given.

Table 1. Overview of randomised controlled trials that tested interventions with exercise in older adults on the four important determinants of muscle quality (1985-2020).

	Type II fibre size		**Mitochondrial functioning**		**Intramuscular adipose tissue**		**Neurological activation**	
	RCTs with positive effect (increase)	RCTs with no effect	RCTs with positive effect (improvement)	RCTs with no effect	RCTs with positive effect (decrease)	RCTs with no effect	RCTs with positive effect (increase)	RCTs with no effect
Exercise Intervention								
Resistance	N=13 [36],[47],[37],[38] [48],[39][b],[41], [42,43],[40],[44], [45],[46]	N= 13 [49],[50],[58],[51] [52],[53],[54],[59] [55],[60],[61],[57] [56]	N=2 [105],[45]	N=4 [106],[107], [108], [109]	N=6 [110],[111], [112],[113], [114],[91]	N=5 [115],[116],[117], [118],[119][d]	N=7 [120],[121],[122], [123],[124],[125], [126]	N=4 [127],[128], [43], [129]
Endurance		N=4 [130][b],[131][c],[50], [43]	N=6 [132][c],[133][c], [131][c],[105], [134],[106]	N=2 [130][b],[135][d]	N=1 [136]	N=1 [110]		N=1 [43]
Concurrent		N=1 [43]	N=1 [106]		N=1 [137],[136]		N=1 [137]	N=1 [43]
High intensity interval or power training			N=3 [108],[135][d], [140][c]				N=3 [125],[138],[139]	N=1 [129]
Electro-stimulation		N=4 [141][c],[142][a], [143][a][144][a]						
Whole-body vibration training								N=1 [145]

All randomised controlled trials (RCTs) reported are performed in older adults without diseases, unless reported as: a, Chronic obstructive pulmonary disease; b, Peripheral arterial disease; c, chronic heart failure; d, diabetes mellitus type II.

Table 2. Overview of randomised controlled trials that tested interventions with nutrition in older adults on the four important determinants of muscle quality (1985-2020).

	Type II fibre size		**Mitochondrial functioning**		**Intramuscular adipose tissue**		**Neurological activation**	
	RCTs with positive effect (increase)	RCTs with no effect	RCTs with positive effect (improvement)	RCTs with no effect	RCTs with positive effect (decrease)	RCTs with no effect	RCTs with positive effect (increase)	RCTs with no effect
Nutritional supplementation								
Milk protein	N=1 [63]	N=4 [146],[147],[44], [60]						
Casein		N=1 [148]						
Animal protein		N=1 [149]				N=1 [150]		
Whey protein		N=2 [151],[46]			N=2 [91],[92]			
Leucine		N=1 [64][d]						N=1 [152]
Vitamin D		N=2 [66],[65]			N=2 [91],[92]			
N-3 PUFA	N=1 [57]	N=1 [153][c]		N=1 [153][c]			N=1 [104]	
Selenium repletion		N=1 [154]						
Iron repletion			N=1 [155][c]					
Milk fat globule membrane							N=1 [103]	

All randomised controlled trials (RCTs) reported are performed in older adults without diseases, unless reported as: a, Chronic obstructive pulmonary disease; b, Peripheral arterial disease; c, chronic heart failure; d, diabetes mellitus type II.

Table 3. Overview of randomised controlled trials that tested interventions with bio-actives in older adults on the four important determinants of muscle quality (1985-2020).

	Type II fibre size		**Mitochondrial functioning**		**Intramuscular adipose tissue**		**Neurological activation**	
	RCTs with positive effect (increase)	RCTs with no effect	RCTs with positive effect (improvement)	RCTs with no effect	RCTs with positive effect (decrease)	RCTs with no effect	RCTs with positive effect (increase)	RCTs with no effect
Bio-active supplementation								
Antioxidants		N=1 [67][a]						
Resveratrol	N=1 [79]		N=2 [77],[79]					
Creatine		N=2 [53],[55]					N=1 [102]	
Chromium picolinate		N=2 [37],[52]						
Nitrate			N=1 [80]					
Epicatechin			N=1 [78]					

All randomised controlled trials (RCTs) reported are performed in older adults without diseases, unless reported as: a, Chronic obstructive pulmonary disease; b, Peripheral arterial disease; c, chronic heart failure; d, diabetes mellitus type II.

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Chapter 2

Relative validity and reliability of isometric lower extremity strength assessment by using a hand-held dynamometer in older adults

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Submitted

Abstract

Background Handheld dynamometry (HHD) is a practical alternative to traditional testing of lower extremity strength. However, its reliability and validity across different populations and settings are not clear. The purpose of this study was to assess the validity and reliability of hand-held dynamometry to assess lower-extremity strength in a population of older adults.

Methods This study included 254 older adults (≥65 years). Isometric knee extension and flexion force were measured by one examiner, using a HHD (n=222), including three repetitions to calculate within-day intra-rater reliability. These measurements were repeated by the examiner in a subgroup (n=23) to analyze intra-rater reliability over a test-retest period of on average 8 weeks. In addition, HHD force measures were performed by a second examiner (n=29) to analyze inter-rater reliability. In another subgroup (n=77), isometric knee extension and flexion torque were measured by one examiner by both the HHD and Biodex System 4 to assess relative validity.

Results HHD and Biodex measurements were highly correlated and showed excellent concurrent validity. HHD systematically overestimated torque as compared to Biodex. Same day intra-rater intra-class correlation coefficients (ICCs) ranged from 0.97 to 0.98. Inter-rater reliability ICCs ranged from 0.83 to 0.95.

Conclusions HHD represents a reliable and valid alternative to Biodex to rank individuals on leg strength, or to assess within-person changes in leg strength over time. Comparing hand-held dynamometry readings to cut-offs or normative values should be done with caution due to systematic overestimation.

Introduction

During the aging process, progressive reduction in skeletal muscle strength occurs. Reduced muscle strength is a predictor for functional outcomes, independent of loss of muscle mass [1, 2]. The age-related decline in muscle strength is more pronounced in the legs than in the arms [3]. Therefore, assessment of lower extremity strength in older adults is increasingly recognized as important [4]. However, traditional testing of lower extremity strength requires costly equipment (e.g. Biodex system) and experienced staff (e.g. physical therapists). Isometric leg strength testing using a hand-held dynamometer (HHD) may offer an alternative as it is less costly and can be applied in various clinical settings.

Several studies have evaluated the accuracy of the HHD to assess leg strength in older people [5-11]. However, in these studies sample sizes were small and different measurement set-ups were used. Earlier studies advocated measuring in a supine position [9], with belt stabilizing [12] and with angle fixation [13]. Increasing the complexity of the set-up did result in higher validity and reliability, but it also diminished the main advantage of the HHD: its feasibility or rather practical applicability. Therefore, the purpose of this study was to assess the validity and reliability of HHD measurements in a practical set-up to assess leg strength in a large population of older adults.

Methods

Study population

This study presents a cross-sectional analysis of data collected for an intervention trial, registered at clinicaltrials.gov as NCT02349282. In that trial [14], isometric knee flexion and -extension strength were measured by HHD in 500 community dwelling older adults, during eligibility screening. We used data of all participants that were eligible for the intervention trial (*n*=77), plus data of the largest subsample of participants who were measured by one examiner during the screening session (*n*=222, of which *n*=41 are also included in the *n*=77), resulting in a total of *n*=258 participants. The 77 participants eligible for the intervention trial attended a second measurement day, where isometric knee flexion and -extension strength were measured by HHD and Biodex.

Eligibility screening was attended by older adults (≥65 years) who estimated their strength to be below-average. Of these participants, 32.4% (*n*=84) had a hand-grip strength below the cut-off level for frailty[15]. For activity and gait speed, respectively 7.3 (*n*=19) and 3.5% (*n*=9) scored below the frailty cut-off level. Criteria for subsequent inclusion in the intervention were: age ≥65 years, BMI of 18.5-30 kg/m^2, vitamin D status 20-50 nmol/L, physically frail or pre-frail based on criteria of Fried *et al.* [15], free of medical conditions or medication use interfering with vitamin D metabolism, and consuming a maximum of 21 alcoholic units per week.

Between December 2014 and June 2015 measurements were carried out at Wageningen University and Hospital Gelderse Vallei in Ede, the Netherlands. The Medical Ethics Committee of Wageningen UR approved the study protocol and all participants gave their written informed consent.

Examiners

Measurements by three examiners were used for the analyses. Examiner A and B were both males, examiner C was female. None of the examiners had prior experience with HHD measurements, but were trained to perform the standardized operation procedure before the first measurements.

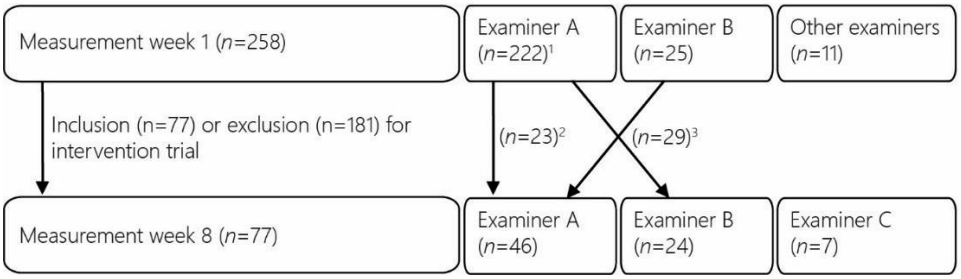

Figure 1. Flow chart of study design and origin of subsamples.
Subsamples used for analysis: 1, same-day intra-rater; 2, 8 week interval intra-rater; 3, Inter-rater; 4, Relative validity.

Figure 1 presents an overview of the measurements that were used for the analyses. Examiner A measured knee-extension and flexion strength in 222 participants at measurement day one. These measurements were used to calculate same day intra-rater reliability. Out of these 222 participants, 23 were measured by the same examiner at measurement day two, and this subsample was used to calculate Δ8-week intra-rater reliability. The calculations of the inter-rater reliability were based on a sample of 29 participants who were measured by examiner A on day one and examiner B on day two, or vice versa. Finally, relative validity was assessed by using the measurements of all 77 participants who attended measurement day two. These measurements were carried out by examiners A, B and C.

Measurements

Hand-held dynamometer

Peak isometric knee- flexion and extension forces were used as the strength measures. Force was assessed in Newton (N), using the MicroFET 2 hand-held dynamometer (Hoggan Health, West Jordan, UT, USA). The subjects were asked to sit upright on an examination table with their knees in a 90˚ angle. Peak force was measured three times per leg with 5 s of muscle contraction and 60 s of rest between repetitions. Subjects were instructed to gradually increase their force when the examiner counted '3-2-1, go' to a peak force until the examiner instructed to stop. All participants started with one rehearsal measurement to ensure full understanding of instructions. Consistent verbal encouragement was provided by the examiner during the 5 seconds of muscle contraction. Stabilization of examiner and HHD position was ensured by measuring while seated against a wall and by offering counter force with both hands. On measurement day two, tests were performed on the Biodex chair instead of an examination table. Besides this difference, other conditions were equal, with participants being seated without the use of chair straps, at an equal height, and at an equal distance of the wall that was used to support the examiner.

Biodex dynamometer

In a subgroup of 77 subjects, peak isometric knee flexion and extension torque were assessed in Newton meter (Nm) using the Biodex System 4 (Biodex Medical Systems, Shirley, NY, USA). Subjects performed the test in a sitting position while holding the handgrips. Chair straps were used to stabilize the subjects to prevent accessory movements. The dynamometer was positioned with the lever arm immediately adjacent to the participant's lower leg with the axis of the dynamometer aligned with the lateral epicondyle of the knee and flexed in a 60˚ angle. Peak torque of flexion- and extension was measured 3 times per leg with 5 s of muscle contraction and 60 s of rest between repetitions. Consistent verbal encouragement and visual feedback on the computer monitor were given to the subjects by the examiners.

Anthropometrics

Bodyweight was measured using a calibrated analogue scale (SECA 716, Hamburg, Germany), without wearing heavy clothing. Weight was reported to the nearest 0.5 kg. Total height was measured to the nearest 0.1 cm using a stadiometer (SECA 213, Hamburg, Germany). Lever length was measured in the validity subsample, using a measuring tape, as the distance in meters between the lateral epicondyle of the femur and the lateral malleolus of the right leg.

Statistical methods

Participants' characteristics are reported as mean with standard deviation (SD), or percentages of the total study group. Agreement (concurrent validity) between the HHD and the Biodex measurements was assessed by Pearson-correlation coefficients, intra-class correlation coefficients (ICC) and Bland-Altman plots. The significance of the difference between HHD and Biodex measurements was assessed by paired sample t-tests. In order to compare HHD and Biodex measurements, HHD force values were multiplied by lever length to convert into torque values. The peak torque of the three attempts was used for analysis. To assess the intra-rater and inter-rater reliability of the HHD method, ICCs and standard errors of measurement (SEM) were calculated. ICCs were calculated via a two-way random effect model, and the SEM was expressed as the product of the standard deviation of the first HHD measurements and the square root of (1-ICC). When ICCs were below 0.5, reliability was considered 'poor', between 0.5 and 0.75 'moderate', between 0.75 and 0.9 'good', and above 0.9 'excellent'. Data analyses were performed with the statistical program SPSS, version 23 (IBM, Armonk, NY, USA). Graphs were created using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA).

Results

Population characteristics are presented in **Table 1**. The mean ± SD age of the total study population was 73.0 ± 5.9 y and 58.3% were men. Mean body mass index was 27.3 ± 3.6 kg/m^2. The mean peak knee-extension and flexion force, measured by HHD were 345±108 and 205±70 N, respectively. The mean lever length in the group that attended the second measurement day was 0.42±0.03 m, and mean peak torques as measured by Biodex were 130±41 Nm for knee extension and 66±21 Nm for knee flexion.

Table 1. Participants' characteristics

	Total population (*n*=259)	Validity subsample (*n*=77)
Demographics		
Gender, % men (n)	58.3 (151)	55.1 (43)
Age, y	73.0 ± 5.9	74.1 ± 6.3
BMI kg/m^2	27.3 ± 3.6	27.9 ± 3.6
Lever length, m	-	0.42 ± 0.03
Biodex torque, Nm (Right leg)		
Peak knee-extension	-	130 ± 41
Peak knee-flexion	-	66 ± 21
MicroFET force, N (Right leg)		
Peak knee-extension	355 ± 108	334 ± 109
Peak knee-flexion	205 ± 70	181 ± 56

Validity

The HHD and Biodex measurements were highly correlated (**Table 2, Figure 2**). The Bland-Altman plots in **Figure 3** show the difference between the Biodex and HHD outcomes. Overall, the HHD measurements significantly overestimated torque as compared to the Biodex (**Table 2**), but Bland-Altman plots revealed no strength-related bias. Differences between HHD and Biodex measurements were more pronounced in the right leg and in the knee flexion measurements. The magnitude of torque overestimation by HHD differed between examiners.

HHD measurements of examiner A (in *n*=45) overestimated torque on average by 9.2% (95% CI: 3.4 – 15.0%, P=0.002), and HHD measurements of examiner B (in *n*=24) overestimated torque on average by 18.5% (95% CI: 12.7-24.3%, P<0.001). This

Table 2. Means, correlation and difference between torque measures obtained by Biodex and MicroFET in older adults (n=77)

	Biodex	*MicroFET*			
	Mean (SD)[a]	**Mean (SD)[a]**	**ICC[b] (95% CI[c])**	**Pears-ons *r***	**Difference (95% CI[b])**
Peak knee-extension, right (Nm)	130.3 (41.2)	140.0 (50.0)	0.85 (0.76-0.91)	0.77*	9.7 (2.4-17.0)*
Peak knee-extension, left(Nm)	130.1 (45.1)	135.8 (54.1)	0.94 (0.90-0.96)	0.90*	5.7 (0.25-11.2)*
Peak knee-flexion, right (Nm)	65.6 (21.2)	75.5 (25.2)	0.79 (0.57-0.89)	0.73*	9.9 (6.0-13.9)*
Peak knee-flexion, left (Nm)	63.5 (21.3)	70.2 (24.5)	0.88 (0.77-0.93)	0.82*	6.6 (3.5-9.8)*

[a] SD = standard deviation, [b] ICC = intra-class correlation coefficient, [c] CI = confidence interval.
*P<0.05

difference in overestimation was not statistically significant (P=0.183) The HHD measurements of examiner C (in *n*=7) underestimated Biodex measurements by 6.1% (95% CI: -36.2% – 23.9%, P=0.635), which was significantly different to examiner B (p=0.012), but not to examiner A (p=0.159).

Reliability

The intra-rater reliability of the HHD measurements performed by one examiner is presented in **Table 3**. Same-day test re-testing resulted in ICCs between 0.97 and 0.98. Peak force measures performed 54±9 days apart resulted in somewhat lower ICCs, with 0.96 for the extension measurements and 0.90 for the flexion measurements. **Table 3** also shows the inter-rater reliability of the HHD measurements. When comparing the test performance of the two examiners, examiner B resulted on average in higher force outputs, with significantly higher scores for left knee extension (difference of 24 N, P=0.033). The ICCs between the tests performed by the examiners were higher for the extension measures (ICC 0.93 and 0.95) compared to the flexion measures (ICC 0.83 and 0.89).

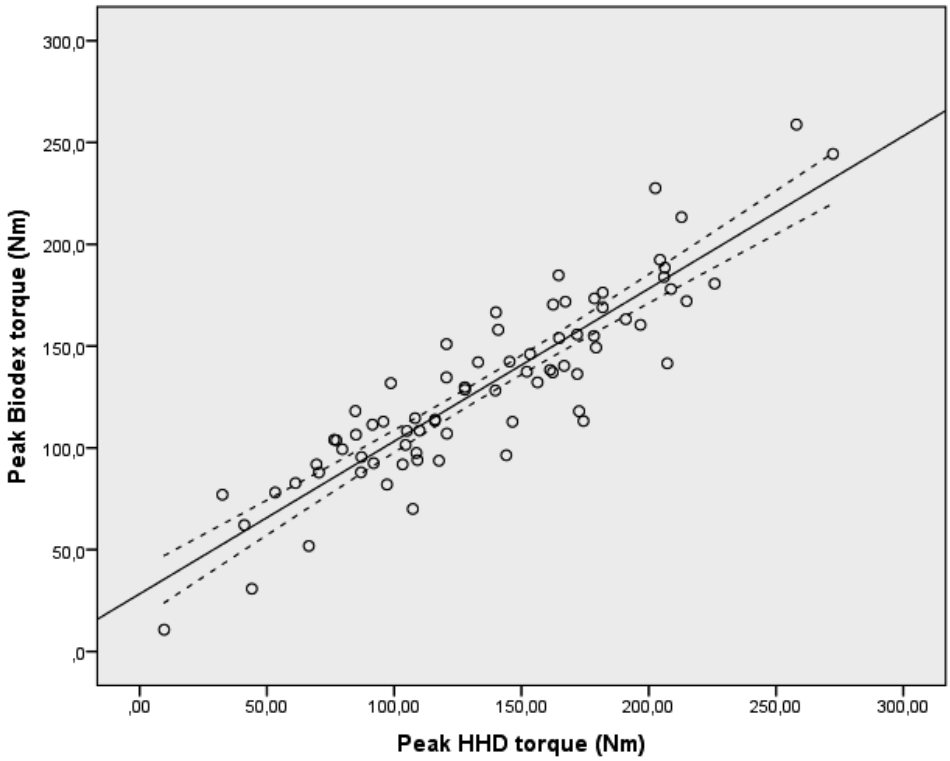

Figure 2. Correlation between Biodex and HHD measurements of left knee extension peak torque (r=0.90, *n*=77). Line of best fit (Biodex= 28.3 + 0.75 × HHD) with 95% confidence interval.

Table 3. Intra-rater reliability for force measures obtained by MicroFET (same-day test-retest period, n=222, 8-week test-retest period, n=23) and inter-rater reliability for force measures obtained by MicroFET in older adults (2 examiners, n=29).

Same day intra-rater	**Mean ± SD[a]** *Trial 1*	**Mean ± SD[a]** *Trial 2*	**Mean ± SD[a]** *Trial 3*	**ICC[b] (95% CI[c])**	**SEM[d]**
Knee-extension, right (N)	347 ± 105	343 ± 104	344 ± 105	0.98 (0.97-0.98)	15
Knee-extension, left (N)	315 ± 106	314 ± 101	314 ± 102	0.97 (0.97-0.98)	18
Knee-flexion, right (N)	194 ± 70	200 ± 67	201 ± 70	0.98 (0.98-0.99)	10
Knee-flexion, left (N)	179 ± 68	183 ± 65	186 ± 64	0.98 (0.97-0.98)	10
Δ8-week intra-rater	**Mean ± SD[a]** *Trial week 0*	**Mean ± SD[a]** *Trial week 8*		**ICC[b] (95% CI[c])**	**SEM[d]**
Peak knee-extension, right (N)	330 ± 117	332 ± 102		0.96 (0.91-0.98)	21
Peak knee-extension, left (N)	297 ± 120	312 ± 113		0.96 (0.90-0.98)	21
Peak knee-flexion, right (N)	183 ± 53	173 ± 46		0.90 (0.78-0.96)	22
Peak knee-flexion, left (N)	167 ± 55	161 ± 46		0.90 (0.76-0.96)	22
Δ8-week inter-rater	**Mean ± SD[a]** *Examiner A*	**Mean ± SD[a]** *Examiner B*	**Difference (95% CI[c])**	**ICC[b] (95% CI[c])**	**SEM[d]**
Peak knee-extension, right (N)	331 ± 111	342 ± 114	11 (-10.5-33.0)	0.93 (0.85-0.97)	28
Peak knee-extension, left (N)	317 ± 131	341 ± 136	24 (2.1-45.6)*	0.95 (0.87-0.98)	24
Peak knee-flexion, right (N)	199 ± 64	198 ± 50	-1 (-18.3-15.6)	0.83 (0.63-0.92)	29
Peak knee-flexion, left (N)	179 ± 65	189 ± 53	11 (-3.0-25.2)	0.89 (0.76-0.95)	23

[a] SD = standard deviation, [b] ICC = intra-class correlation coefficient, [c] CI = confidence interval, [d] SEM = standard error of measurement. *P<0.05.

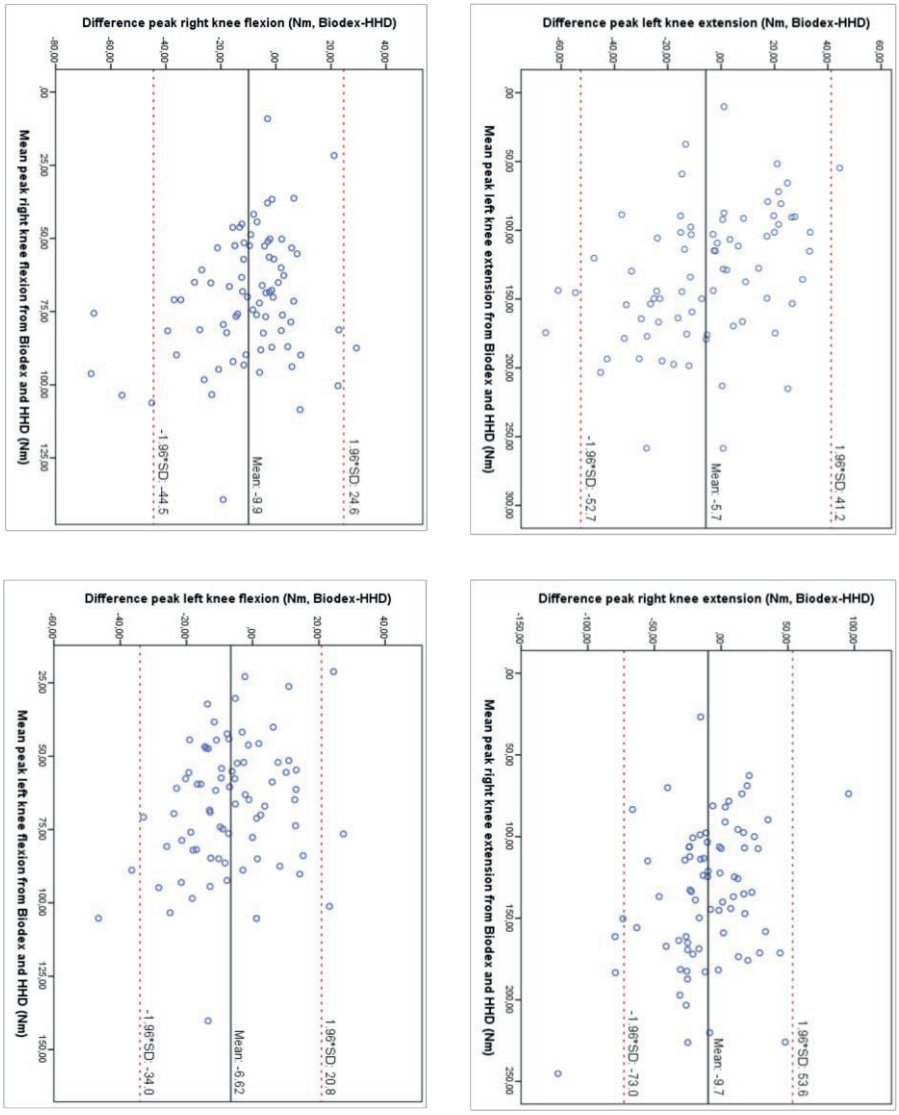

Figure 3. Bland-Altman plots Biodex versus MicroFET measurements (*n*=77)

Discussion

In this study, we tested the relative validity of knee-extension and flexion torque measured by handheld dynamometry (HHD) as compared to Biodex dynamometry. Besides, we assessed the intra-rater and inter-rater reliability of isometric knee-flexion and extension force assessment by handheld dynamometry. We observed strong correlations between HHD and Biodex torque measurements. However, HHD significantly overestimated torque, which was most pronounced for isometric knee flexion measurements. This overestimation is unexpected, since the greater measurement angle of the leg (90º in HHD vs 60º in Biodex), and the absence of trunk stabilization straps during HHD measurements, have both been reported to decrease peak torque [16, 17]. Previously performed studies comparing Biodex and HHD have indeed found lower values for HHD measurement [6, 9]. The overestimation of torque measured by HHD was systematic and equal over the range of measures levels of torque. This suggests that calibration of HHD readings to Biodex readings should be possible, and that caution is warranted when HHD readings are being compared to cut-off values based on Biodex measurements.

The overestimation of torque measured by HHD was more explicit for knee flexion than for knee extension measurements. Knee flexion measurements also scored worse than knee extension on inter-rater reliability, and on intra-rater reliability when measured 8 weeks apart. This is in line with the study of Martins et al. [18], who found higher validity and intra-rater validity for knee extension than for knee flexion in a young population. It is important to note that although knee flexion assessment performs worse than knee extension assessment, still good validity (ICC 0.79-0.88), good inter-rater reliability (ICC 0.83-0.89) and excellent intra-rater reliability (ICC 0.90-0.98) were observed.

The systematic overestimation should not hamper ranking of individuals and assessment of within-person changes over time. After all, we found excellent intra-rater reliability of HHD measurements with an 8-week break in between (ICCs 0.90-0.96), even though the test conditions were slightly different. In a similar population, Schaubert et al. [19] also found excellent intra-rater reliability (ICCs 0.92-0.93) for HHD measurements separated by a 6-week period. Intra-rater reliability of HHD measurements on the same day was even higher (ICCs 0.97-0.98). These values are comparable to those found by Wang et al. (0.98-0.99) [11] but higher than values reported by Katoh et al. (0.88-0.91) [8]. Katoh et al. did not include a familiarization

trial, and performed only two HHD measurements per leg. The lack of a familiarization trial forms a likely explanation of lower intra-rater reliability, and indicates the importance of including such a familiarization trial for HHD measurements.

We observed good to excellent inter-rater reliability for the assessment of knee extension and flexion force. However, we did find differences between examiners. Examiner B measured higher forces with HHD than examiner A did, which reached significance for left knee extension force. Compared to Biodex readings, torque overestimation was twice as high for HHD measurements performed by examiner B compared to those performed by examiner A. Both examiners were similarly trained, used the same technique, were similarly experienced with HHD measurements, were both male, and were of similar age. The difference in measured HHD force between these two examiners might be due to factors beyond these similarities, such as strength of the examiner [20]. This is in line with our observation that the HHD measurements performed by the female examiner C in 7 participants showed signs of underestimation compared to Biodex.

The main strength of this study is the large sample size. Other strengths are the assessment of intra-rater reliability on the same day and with an 8-week interval. Additionally, our use of a convenient and replicable testing set-up and examiner training supports the main advantage of the HHD: its low practical and financial burden to use. Some limitations should be considered when interpreting the results. First, HHD testing positions on measurement days one and two were slightly different. Likely, this led to lower reliability than when measurements would have been compared in identical positions. Second, despite allowing short periods of rest between tests, fatigue may have occurred on the measurement days, resulting in lower readings over time, and thus lower reliability. Finally, knee angles for HHD and Biodex were different (90º vs 60º), which might have had an effect on validity results.

In conclusion, hand-held dynamometry performed by well-trained examiners in a stable position represents a reliable and valid alternative to Biodex to assess lower extremity strength in an older population, allowing for ranking of individuals, or for assessing within-person changes over time. Comparing hand-held dynamometry readings to Biodex-based cut-offs or normative values should be done with caution due to systematic overestimation.

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Chapter 3

A 4-week exercise and protein program improves muscle mass and physical functioning in older adults - A pilot study

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Experimental Gerontology. 2020 Sep 17;141:111094

Abstract

Background Prehabilitation might attenuate common surgery-induced losses in muscle mass and physical performance. Beneficial effects of physical exercise with protein supplementation have been reported in older adults multiple times, but typically after an intervention of at least 12 weeks. The time-window for pre-surgery training is often limited to around 30 days, and it is not known if it is possible to achieve comparable results in such a short time window.

Objectives The aim of this study was to pilot-test the effectiveness of an intense four-week combined exercise and protein supplementation program on skeletal muscle-related outcomes in a Dutch older adult population.

Methods This study was a one-armed pilot trial. Seventeen older sedentary men and women, aged 55-75y, not scheduled for surgery underwent a 4-week intervention program consisting of a twice-weekly supervised resistance and high-intensity aerobic exercise training of 75 min, combined with daily protein supplementation (31 g). After two and four weeks, isometric quadriceps maximal voluntary contraction (MVC) was assessed via Biodex and quadriceps cross-sectional area (CSA) via magnetic resonance imaging. Other outcome measures were handgrip strength, chair rise time and maximal aerobic capacity (VO_2-max), as assessed from a submaximal exercise test.

Results Compliance to the training sessions (99.3%) and the protein supplementation (97%) was very high. The 4-week exercise and protein program improved isometric quadriceps MVC in the dominant leg (Δ14 ± 4 Nm, P=0.001) and in the non-dominant leg (Δ17 ± 5 Nm, P=0.003). Quadriceps CSA increased with 2.3 ± 0.7 cm^2 (P=0.008) in the dominant leg and with 3.2 ± 0.7 cm^2 (P<0.001) in the non-dominant leg. VO_2-max improved with 3.3 ± 1.1 ml/min/kg (P=0.014) and chair rise test with -3.8 ± 0.5 s (P<0.0001). We observed no changes in body weight and handgrip strength.

Conclusion A 4-week exercise and protein intervention led to improvements in muscle-related outcomes in sedentary older adults.

Introduction

Surgery largely impacts the physical status of patients [1]. A surgical procedure induces a stress response, which alters the functioning of different organs and physiological systems [2]. The surgery-induced stress response is thought to serve as a protective mechanism, as it enables the body to provide the essential substrates needed for the healing process [3]. In order to provide these substrates, catabolic processes are activated which lead to an increase in protein breakdown and consequent loss of muscle mass [4-6]. The surgery-induced loss of muscle mass can have serious consequences on the daily functioning of older patients, who often already experience a certain degree of catabolism before surgery [4, 7, 8]. Older adults with a compromised physical status before surgery are especially at risk of negative post-surgery outcomes such as prolonged hospitalization, higher risk of infections, and death [9-11].

The number of older adults undergoing surgery is steadily increasing due to changing demographics [12]. In the case of elective or semi-elective surgery, the surgery is planned and can be prepared for. This offers possibilities for physical training prior to surgery, a concept described in the literature as prehabilitation or "better in, better out" [13]. The goal of this physical preparation is to improve the functional capacity of an individual to withstand the stressor of the surgical procedure [14]. A prehabilitation program typically consists of a multimodal approach in which aerobic exercise, resistance training, and increased protein intake are combined to employ their synergetic effects on muscle mass and physical performance [8]. Recently, systematic reviews by moran et al. [15] and hughes et al. [16] concluded that prehabilitation can improve postoperative outcomes, but that there is a need to explore the potential for interventions that achieve maximum improvements within 30 days.

The short time window of around 30 days in which patients can be trained before surgery forms the main threat to the success of prehabilitation. Regarding older adults, it is shown in multiple studies that relevant improvements in lean body mass and strength can be achieved after a training period of around four months [17, 18]. It is not yet known if one month of training can already induce relevant benefits for this population. Therefore, before the further implementation of prehabilitation in older adults is warranted, we need to know whether positive results on muscle mass and physical performance can be achieved in such a short time window. The aim of the present study is to assess the effects of an intense four-week combined exercise and protein supplementation program on muscle strength, muscle mass, and aerobic capacity in older adults.

Methods

Study design and participants

This 4-week single-arm repeated measurements pilot-study included 18 untrained healthy, sedentary men and women who were not scheduled for surgery. Potential participants were recruited from a volunteer database from Wageningen University and Research, and eligibility was assessed via five questionnaires: (1) a screening questionnaire regarding the inclusion and exclusion criteria, (2) a screening questionnaire regarding the demographics of the participants, (3) the Short Questionnaire to Assess Health Enhancing Physical Activity (SQUASH) [19] to assess current activity level, (4) the Physical Activity Readiness Questionnaire (PARQ) and (5) a Magnetic Resonance Imaging (MRI) screening questionnaire.

Eligible participants were those between 55 to 75 years, who only performed physical activity for a maximum of 30 minutes on five days per week, and who did not participate in a structured exercise program the last three months before recruitment. Exclusion criteria were; having an allergy for (or being sensitive to) milk proteins, being lactose intolerant, being diagnosed with renal insufficiency (estimated glomerular filtration rate <60 ml/min/1.73 m2), being diagnosed with cancer for which currently treated, having a diet which affects protein intake, having a contraindication for MRI scanning or exercise training, participating in another intervention trial, not being able to understand Dutch, and not having a general physician.

Study outcome assessment (at week 0, 2 and 4) and supervised training sessions were performed at hospital Gelderse Vallei in Ede, The Netherlands, between April 2016 and July 2016. The study was approved by the medical research ethics committee and is registered at The Netherlands National Trial Register (NTR5701). For each participant, informed consent was obtained.

Intervention

The intervention consisted of an exercise program and protein supplementation. The exercise program contained both supervised exercise training sessions and unsupervised home-based exercise guidelines. The supervised exercise training of approximately 75 minutes took place at the department of physical therapy of the Gelderse Vallei Hospital in Ede and was given twice a week. An experienced

physiotherapist supervised the training sessions. During these training sessions, the participants performed 30 minutes of resistance training followed by 30 minutes of high-intensity interval aerobic training. Resistance training included the following exercises: Leg Press (Technogym, Rotterdam, The Netherlands), Leg Extension, Latissimus Dorsi (Lat) Pulldown, and Chest Press (Lifefitness, Barendrecht, The Netherlands). All exercises were performed in sets of three, with 12 repetitions each set and with one-minute rests between the sets. Resistance exercises were performed at 12-Repetition Maximum (12-RM) with a perceived exertion rate between 13-15 on the Borg-scale [20].

High-intensity interval aerobic training was performed on a cycle-ergometer (Ergoline, Bitz, Germany) and consisted of four high-intensity bouts at 90% of the calculated heart rate reserve (HRR) and a perceived exertion rate of 15-17 on the Borg scale. Each bout lasted one to two minutes and was alternated by three minutes of cycling at a lower intensity of 50-60% of the calculated HRR and perceived exertion rate between 11-13 on the Borg-scale. All training sessions included a 10-minute warm-up and a five-minute cool down on a cycle-ergometer with an intensity of 20-30 Watt (W) at 65-80 rotations per minute (rpm). Between the supervised training sessions, there was a rest period of at least 48 hours.

With regard to the home-based exercise guidelines, participants were instructed to be moderately active for a minimum of 30 minutes on all days that no supervised training was performed. The 30 minutes could be divided into parts of at least 10 minutes. Moderate intensity was defined as a perceived exertion between 12 and 15 on a Borg scale. During the intervention period, participants were instructed to register their daily physical activity in a diary with the corresponding perceived exertion on a Borg scale.

During the intervention period, all participants consumed 250 ml of a protein supplement (FrieslandCampina Consumer Products Europe, Wageningen, The Netherlands) twice a day in addition to their habitual diet. Per portion, the protein supplements contained 397.8 kJ, 15.5 grams of protein (milk protein concentrate, consisting of 80% protein (MPC-80)), 7.3 grams of lactose and 0.5 grams of fat. The participants ingested the protein supplements directly after breakfast and lunch. On supervised training days, the participants consumed the protein supplements within 30 minutes after training instead of directly after lunch. To monitor compliance, participants were instructed to return empty bottles and to record supplement intake on a calendar.

Outcome measurements

Quadriceps strength, expressed as the maximal voluntary contraction (MVC), was assessed via isometric dynamometry (Biodex System 3, Biodex Medical Systems, New York). Participants were seated with their knees flexed at 60 degrees, stabilised by straps at the hip, shoulders, and upper leg to prevent unwanted movements. Participants performed one submaximal contraction to familiarise with the technique, followed by three 5 s MVC's with 30 s rest between contractions for each leg. The highest torques (Nm) of the dominant and non-dominant leg were used for analysis.

Quadriceps mass, expressed as quadriceps cross-sectional area (CSA), was measured using MRI scanning with a 3-T Siemens Magnetom Verio Syngo MR B19 MRI scanner (Siemens AG, Erlangen, Germany). Participants lay supine in the MRI scanner, with a vitamin E capsule taped on the right leg at the midpoint between the iliac crest and the upper edge of the patella. The vitamin E capsule was used as a mark during the analysis of the MRI images. A body coil was placed over the legs, with the midpoint of the coil above the vitamin E marker. During each measurement, both upper legs were imaged. The MRI scanner used 2D spin-echo pulse sequences to obtain T1-weighted images (TR: 700 ms, TE: 10 ms, flip angle: 140 degrees, turbo factor: three, acquisition time: three minutes). Eventually, 50 slices of five mm were obtained for each leg, and for each leg, the slice in which the vitamin E capsule was best visible was selected for analysis. Analysis of the MRI images (TIFF format, 16-bit grayscale) was performed in the software program ImageJ [21] by one analyst. Quadriceps muscle tissue was manually separated from other muscles and from other tissues. When necessary, a threshold was manually set to obtain a clear border between quadriceps muscles and other tissues. After all area measurements were performed, the first six measurements were discarded and repeated by the same analyst to reduce possible learning-induced intra-observer variation. A second observer re-analysed a random subgroup of eleven images to assess inter-observer variation as well, which was less than five per cent between both observers. In this way, from all measurement time points, mid-quadriceps CSA in cm^2 of both legs was assessed and used for analysis.

Muscle quality was expressed as torque per cm^2 of quadriceps CSA and was calculated for both legs by dividing peak torque by quadriceps CSA. Quadriceps power was assessed via the 10-times chair rise test on a 45-cm high chair [22]. Handgrip strength (kg) of both hands was measured with the use of a hand

dynamometer (Jamar®, Jackson, Michigan) [23], in a seated position with the elbow flexed at 90 degrees. Participants completed three measurements per hand, and the maximum reading per hand was used for analysis. During the screening procedure, height (m) of each participant was obtained with the use of a stadiometer. In addition, weight (kg) was obtained during each visit with a calibrated analogue scale. Participants were instructed not to wear heavy clothing when weighting. Body mass index (BMI) was calculated as weight (kg) / height $(m)^2$. Physical activity levels were obtained with the use of an accelerometer (PAM AM300, PAM BV). All participants wore the accelerometer during two periods of seven consecutive days: before the start of the intervention period and during the final week of the intervention period. From the results of the accelerometer, the total metabolic equivalent minutes (METmin) per day that contributed to all daily activities were calculated for each participant.

Aerobic capacity, expressed as maximal oxygen consumption (VO_2-max), was estimated with the Åstrand-Rhyming submaximal exercise test on a stationary bicycle (Ergoline, Bitz, Germany) [24]. During a warm-up phase, the initial workload (50 W) was increased every minute by 50 W for males and 25 W for females until a steady heart rate around 120 bpm was achieved. When this steady heart rate was achieved, the 6-minute exercise test started. Heart rate (HR) was recorded every minute with a Polar HR sensor. VO2-max was estimated with the use of the mean HR of the fifth and the sixth minute, the Åstrand-nomogram, and a correction factor for age. If the HR differed more than five beats between minute five and six, the test was prolonged with a maximum of three minutes to achieve a more steady HR. Over the repeated measurements, room and time of day were kept identical for each participant.

Dietary intake was assessed by self-administered web-based 24-hour recalls (Compl-eat, Wageningen University, Wageningen, NL), on two random days in the week before the start of the intervention, and on two random days in the final week of the intervention. Trained dietitians checked the recalls, and calculated total energy and nutrient intake by using the 2013 Dutch food composition database.

Statistical analysis

Sample size (n=18) was calculated by using power calculation software (G*Power, Heinrich-Heine-University, Düsseldorf), by using a two-tailed alpha of 0.05, a beta of 80%, a drop-out rate of 20% and an estimated difference of 20 ± 25 Nm. This difference was estimated by using an expected clinically relevant increase of 10% from normal average maximum isometric knee extension torque of 200 Nm as measured by Biodex in adults aged ≥ 50 years [25].

Changes over time in primary and secondary outcomes were assessed by linear mixed models, with a random intercept and a variance components covariance structure. Time was added as a fixed factor, participant as a random factor and models were adjusted for gender. Model assumptions were checked via visual inspection of QQ-plots and scatterplots. In case of a significant time-effect, Bonferroni-adjusted posthoc analyses were performed. Differences in dietary intake, exercise performance and physical activity levels between baseline and end of follow-up were assessed by paired sample t-tests. All analyses were carried out in SAS 9.4 (SAS Institute Inc.) in per-protocol fashion. Descriptives are presented as mean ± SD or median (p25 – p75), and differences are presented as mean ± SEM.

Results

A total of 18 participants were enrolled in this study (**Figure 1)**. One participant dropped out before the week 2 measurement due to medical conditions unrelated to the study protocol. The remaining 17 participants completed all measurements, but one participant was excluded for the non-dominant knee extension analysis, due to the inability to perform a maximum effort caused by pain complaints. Another participant was excluded from quadriceps cross-sectional analyses due to failed slice identification. Baseline characteristics of the 17 completers are presented in **Table 1**. The study population consisted of an equal proportion of males and females, with a median age of 71 years and a median BMI of 26.0 kg/m^2. Participants were active for, on average, 430 ± 134 METmin per day.

Table 1. Baseline characteristics

	Mean ± SD (unless stated otherwise)
Male, % (n)	53 (9)
Age (y), median (IQR)	71 (69-74)
Body weight (kg)	76.8 ± 9.2
Height (cm)	169 ± 8
BMI (kg/m^2), median (IQR)	26.0 (25.6-28.6)
Physical activity (METmin/day)	430 ± 134
Living situation, % (n)	
Independent, alone	35 (6)
Independent, with roommate or partner	65 (11)
Smoking, % (n)	
Current smoker	6 (1)
Former smoker	53 (9)
Non-smoker	41 (7)

BMI, body mass index; IQR, interquartile range; METmin, metabolic equivalent minutes; SD, standard deviation.

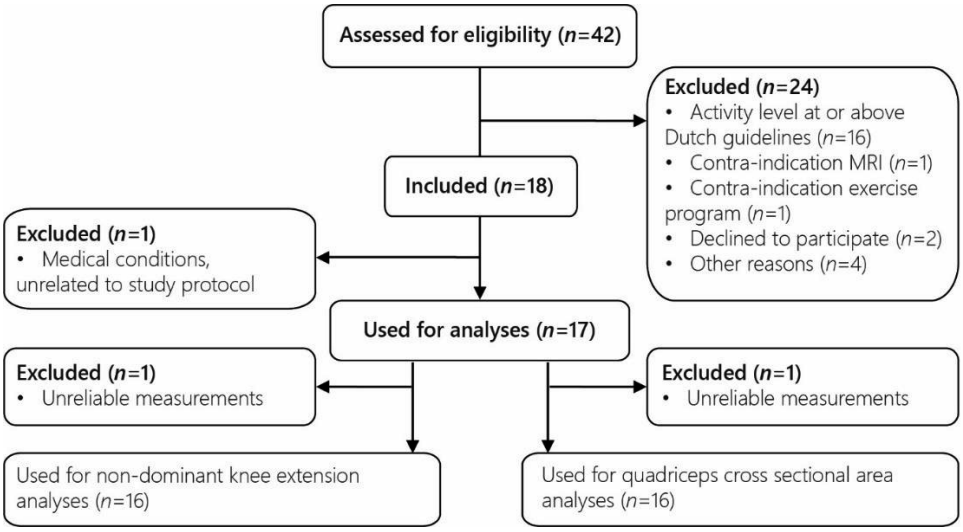

Figure 1. Flowchart of participants

Changes in primary and secondary outcomes are presented in **Table 2.** Isometric knee extension torque increased significantly in the dominant leg (Δ14 ± 4 Nm, P=0.001) and the non-dominant leg (Δ17 ± 5 Nm, P=0.003) between week 0 and week 4 (**Figure 2)**. Quadriceps CSA increased between week 0 and week 4 with 2.3 ± 0.7 cm^2 (P=0.008) in the dominant leg and with 3.2 ± 0.7 cm^2 (P<0.001) in the non-dominant leg (**Figure 2)**. In the non-dominant leg, a significant increase in quadriceps CSA was already observed after two weeks (Δ2.1 ± 0.7 cm^2, P=0.005). The time needed to complete the 10-times repeated chair rise test decreased with 3.8 ± 0.5 s (P<.0001) between week 0 and week 4. Estimated VO_2Max increased with 3.3 ± 1.1 ml/min/kg (P=0.014). Handgrip strength and body weight did not change over the 4 weeks.

Response to the training program showed interesting interindividual variation. Two participants (**Figure 2**, ID=2 and ID=10) showed a decrease in quadriceps CSA in both legs. Another participant (ID=11) showed improvements of 45% in non-dominant knee extension torque, and only 3% in non-dominant quadriceps CSA, indicating a large increase in muscle quality (expressed as torque per cm^2). In the total sample, muscle quality increased only in the dominant leg (time-effect P=0.044).

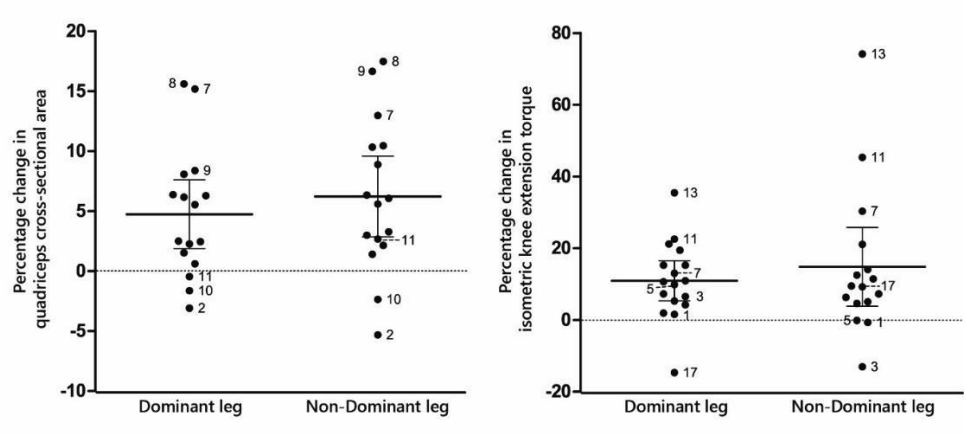

Figure 2. Individual, relative changes in (non-) dominant quadriceps cross-sectional area (left) and isometric knee extension torque (right) from week 0 to week 4. The thick line represents the mean, error bars represent 95% confidence interval. Numbers identify extreme responders (negative responders and responders with >15% change in CSA or >30% change in MVC) and highlight interesting individual data points such as ID=11, who shows improvements in MVC but not in CSA.

Compliance to the supervised exercise sessions was 99.3%. The workload of the leg-press exercise increased on average with 41 ± 3 kg (P<.0001), and the workload of the high-intensity interval training increased with 7 ± 3 Watt (P=0.027). Participants increased their physical activity level on average by 56 ± 24 METmin/day between week 0 and week 4 (P=0.031). On average, participants consumed 54 of the 56 protein supplements (97% compliance, range: 91-100%). Daily protein intake increased from 0.9 ± 0.3 g/kg to 1.2 ± 0.2 g/kg (Δ0.4 ± 0.1 g/kg/d, P<.0001). Protein intake during breakfast increased from 10 ± 8 g to 25 ± 7 g (Δ15 ± 1 g, P<.0001), and protein intake during lunch increased from 19 ± 14 g to 31 ± 11 g (Δ12 ± 4 g, P=0.008). Habitual energy and protein intake, without the energy and protein from the supplements, did not change between baseline and week 4. All participants reported mild muscle ache at least one time during the training period. In most of the cases (61%), the muscle ache disappeared after one day, and in the other cases, the muscle ache disappeared after two (25%) or three (14%) days. Muscle ache represented 49 of all 64 adverse events reported in this study. The other adverse events consisted of falls (n=4), flu-like complaints (n=3), flatulence (n=2), constipation (n=2), joint pain (n=2), back pain (n=1) and light-headedness during the cycling training (n=1).

Table 2. Means of primary and secondary outcomes per visit

	Week 0	Week 2	Week 4	Time-effect
Isometric knee extension (Nm)				
Dominant leg (n=17)	138 ± 11^{a}	144 ± 11$^{ab.}$	152 ± 11^{b}	P=0.004
Non-dominant leg (n=16)	135 ± 9^{a}	145 ± 9^{ab}	152 ± 9^{b}	P=0.004
Quadriceps cross-sectional area (cm^2)	58.4 ± 1.9^{a}	59.5 ± 1.9^{ab}	60.7 ± 1.9^{b}	P=0.010
Dominant leg (n=16)	57.8 ± 1.6^{a}	59.8 ± 1.6^{b}	60.9 ± 1.6^{b}	P<0.001
Non-dominant leg (n=16)				
Muscle quality (Nm/cm^2)				
Dominant leg (n=16)	2.3 ± 0.2^{a}	2.5 ± 0.2^{a}	2.5 ± 0.2^{a}	P=0.044
Non-dominant leg (n=15)	2.3 ± 0.2	2.4 ± 0.2	2.4 ± 0.2	P=0.283
Grip strength (kg)				
Dominant hand (n=17)	27 ± 1	28 ± 1	29 ± 1	P=0.204
Non-dominant hand (n=17)	28 ± 2	28 ± 2	29 ± 2	P=0.181
10-times repeated chair rise test (s) (n=17)	20 ± 1^{a}	18 ± 1^{b}	16 ± 1^{c}	P<0.0001
Estimated maximum oxygen consumption (ml/min/kg) (n=17)	29 ± 1^{a}	31 ± 1^{ab}	32 ± 1^{b}	P=0.016
Body weight (kg) (n=17)	75.8 ± 1.9	76.3 ± 1.9	76.4 ± 1.9	P=0.076

Data presented as means ± standard error. Superscript characters represent results of post hoc analyses in case of a significant time-effect. Different letters represent significant differences after Bonferroni adjustment.

Discussion

Prehabilitation programs might be effective to improve postoperative outcomes, but the time-window in which patients can be trained is limited to around 30 days. Therefore, there is a need to explore potential interventions that achieve maximum improvements within this short time window [15]. The present study revealed that a 4-week intervention consisting of supervised resistance exercise and high-intensity interval training, protein supplementation and increased daily physical activity, already leads to substantial improvements in quadriceps cross-sectional area, quadriceps strength and power, and aerobic capacity in older adults.

Our 4-week intervention resulted in improvements in quadriceps strength of 10% in the dominant leg and 12% in the non-dominant leg. Earlier studies in older adults have shown that resistance exercise, with or without protein supplementation, leads to improvements in muscle strength after 12 weeks [26-31]. Two of these studies measured isometric knee extension strength and showed improvements of 12.7% [27] and 14.7% [30] after 12 weeks of training. The reason that we achieved similar improvements in only four weeks might be due to differences in the training program, as these studies either did only use body-weight or TheraBand resistance exercises [27] or did not provide concurrent protein supplementation [30]. While our program was rather intensive, combining resistance exercise and high-intensity interval training, twice daily protein supplementation and increased daily physical activity.

Protein supplementation during the exercise program might be imperative to yield results on muscle strength and function. In a study by Dronkers et al. [32], no improvements in chair rise test performance or VO_2Max were observed, while we observed a 9% improvement in chair rise test performance and an 8% improvement in VO_2Max. Their participants followed an exercise training regimen that was similar to ours but without protein supplementation. Protein supplementation can improve responses to resistance exercise training [33] and early evidence suggests it can also improve responses to aerobic exercise training [34]. Older adults are advised to consume at least 25 grams of protein per main meal to optimally stimulate muscle protein synthesis [35]. Studies show that the majority of older adults do not meet this level during breakfast and lunch [36]. The protein supplementation in our study increased the proportion of participants that reached the 25g threshold from 6% to 41% during breakfast, and from 17% to 65% during lunch.

Besides improvements in quadriceps strength, chair rise test and VO_2Max, we observed improvements in quadriceps CSA of 3.9% in the dominant leg, and 5.4% in the non-dominant leg. To the best of our knowledge, we are the first to report significant increases in muscle mass in older adults after already four weeks of exercise training and protein supplementation. In many studies, changes in muscle mass are assessed for the first time after 10 to 12 weeks of intervention. One study that did report vastus lateralis CSA changes in older adults (n=6) after four weeks of resistance exercise found a similar increase of around 4%, but this increase was not significant, likely due to their limited sample size [29].

The increases in quadriceps CSA that we observed are clinically relevant. A study monitoring muscle atrophy in the first week of hospitalization after elective hip surgery found a 3.4% decrease in quadriceps CSA [37]. In the case of a pre-surgery increase in CSA of 3.9%, as observed in our study, this 3.4% decrease in CSA in the first week after hospitalization would not have resulted in a net quadriceps CSA loss. This means that this prehabilitation program might be successful in preventing net losses in quadriceps CSA in the period of -4 to +1 week around surgery. To further prevent muscle mass loss in the following weeks after hospitalization, post-operative strategies should be added to the prehabilitation program. Post-operative strategies should ideally consist of supplementation of anabolic nutrients which are able to attenuate bed rest-induced muscle atrophy, like protein [38], leucine [39], β-hydroxy β-methyl butyric acid [40], in combination with exercise or exercise mimetics within the patients' possibilities [38].

Our participants showed excellent compliance to the supervised training sessions and the protein supplementation. On the other hand, the compliance to the home-based exercise was low, as indicated by an average increase of only 56 METmin/day, measured in week four of the program. Attending the supervised training sessions of that week alone would have led to an increase of around 100 METmin/day, indicating that participants decreased their unsupervised physical activity. This suggests that increasing activity beyond supervised exercise training and protein supplementation is not necessary to achieve improvements similar to our findings and that short-term training programs should focus on reaching adequate intensity rather than increasing the overall activity volume.

A clear limitation of this study is the lack of a control group. For some outcome measures, we are unable to rule out the possibility of a learning effect. The outcome measures that are most prone to a learning effect are isometric knee extension

strength, chair rise test and handgrip strength. We observed no changes in handgrip strength, so this was probably not affected by a learning effect. Moreover, it is unlikely that the effects found in quadriceps strength and power are solely addressable to a learning effect, as these results are very much in line with the results of quadriceps cross-sectional area, an objective outcome that cannot be influenced by learning effects. The VO2-max test is typically prone to a learning effect. However, we used a heart rate based Åstrand test, in which physiological phenomena influence the estimated VO2-max more than mental determinants.

This study was performed in sedentary, healthy older untrained adults, who were not scheduled for surgery. The extrapolation of our findings to patient populations should be done with caution. Physiological mechanisms that are related to the reason for surgery likely alter the generalisability of our findings from one population to another. Healthy patients, for instance those awaiting knee-replacement, might show responses similar to those in our study, while patients with underlying diseases that influence hormone and cytokine levels might have very different responses to the anabolic stimuli. This notion is illustrated by the work of Boereboom et al., who showed that a 31-day HIIT training improved VO2max in healthy older adults [41], but not in colorectal cancer patients [42]. Future work should endorse these caveats and test prehabilitation programs that are effective in healthy older adults in real patient populations.

To conclude, this study shows that it is possible to improve muscle mass, muscle strength and aerobic capacity already within four weeks of supervised resistance exercise training in combination with protein supplementation. Future studies are needed to confirm the findings of this pilot-study versus a control group, in preoperative patients.

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Chapter 4

A novel oral nutritional supplement improves gait speed compared to standard supplement in older adults with (or at risk of) undernutrition: results from a randomised controlled trial

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Submitted

Abstract

Background Undernutrition in older adults is mainly addressed by oral nutritional supplements, which do not in improve physical functioning. Urgently needed improvements in physical functioning may be obtained via alterations in the formulation of these supplements.

Objective To compare the effects of a novel oral nutritional supplement containing a mixture of whey and casein protein, ursolic acid, additional free branch-chained amino acids and vitamin D, with a standard oral nutritional supplement on body composition and physical function in undernourished older adults.

Methods N=82 older adults (>65y) with (or at risk of) undernutrition (n=82) were randomly allocated to 12 weeks of supplementation with the novel supplement or standard supplement. At baseline, after 6 and 12 weeks, body composition was measured, and blood was collected. Additionally, at baseline and after 12 weeks, physical functioning was measured, and vastus lateralis mRNA and protein expression were assessed.

Results Body weight increased in the 12 weeks, both in the novel supplement group (+1.6 ± 0.2 kg, p<.0001) and in the standard supplement group (+1.8 ± 0.2 kg, p<.0001). Lean mass tended to increase in both groups (+0.3 ± 0.1 kg, P=0.08), without between-group differences. Non-dominant knee flexion increased significantly within the standard supplement group (38 ± 10 N, P=0.003). Knee extension force, handgrip strength and total SPPB score did not change in the two groups. Gait speed during 4m and 400m tests improved over time in the novel supplement group, whereas the standard supplement showed no improvements (time*treatment effects 400m: p=0.038 and 4m: p=0.048). Proteins involved in mitochondrial pathways were upregulated in both treatment arms. Gene sets related to mitochondrial functioning were strongly upregulated in the novel supplement group and downregulated in the standard supplement group.

Conclusions A 12-week intervention with the novel nutritional supplement improved walking performance both during short and long distance as compared to standard supplement, which might be explained by improved mitochondrial functioning.

Introduction

Undernutrition (or malnutrition) is a medical condition that is highly prevalent in older adults [1] and is often accompanied by loss of skeletal muscle mass and impaired physical functioning [2]. Undernutrition is mainly addressed by oral nutritional supplements (ONS). These supplements are often energy and protein dense and contain macronutrients and micronutrients [3]. From pooled results of 62 studies, Milne *et al*. concluded that ONS show a small but consistent increase in body weight [4]. On the other hand, the authors concluded that ONS do not improve physical functioning, and only slightly increase arm muscle circumference, which is a measure of muscle and fat tissue.

The absence of clear effects on muscle mass and physical functioning might be partly the consequence of the current formulation of the supplements. Many ONS contain only casein protein, while whey protein has shown to be superior to casein protein in promoting skeletal muscle anabolism [5, 6]. This superiority of whey over casein is mainly accredited to its higher content of leucine, which by itself can increase muscle protein synthesis on short term [7-9]. However, long-term interventions with only leucine supplementation fail to show beneficial effects on muscle protein synthesis [10]. An explanation for the lack of long-term benefits might be that administration of only leucine decreases plasma concentrations of valine and isoleucine, which makes their availability rate-limiting for muscle protein synthesis [11]. This issue might be circumvented by supplementing a mixture of valine, isoleucine and leucine (together forming the branched-chain amino acids, BCAAs).

Besides whey protein and BCAAs, recent advances in gene expression studies have identified other compounds with promising physiological potential. One of the most prominent compounds is ursolic acid (UA). UA is a pentacyclic triterpenoid molecule that naturally occurs in a variety of fruits and herbs. Data from gene expression studies show that UA downregulates the muscle atrophy-inducing genes Muscle RING finger 1 (MuRF-1) and Atrogin-1, while it upregulates insulin-like growth factor 1 (IGF-1) signalling [12]. In a mouse model, a 5-week diet enriched with 0.27% UA resulted in increased quadriceps weight and grip strength compared to a control diet [12]. In another mouse study, 2-week diets with 80 and 240 mg/kg UA improved endurance capacity [13].

Altering the formulation of ONS might contribute to urgently needed improvements in muscle mass and physical functioning. This suggestion is strengthened by two recent trials that show promising effects on physical functioning by using a whey-based ONS high in leucine and vitamin D [14], or an ONS high in vitamin D and enriched with calcium β-hydroxy β-methyl butyrate [15]. Adding free BCAAs and ursolic acid to the formulation of ONS might result in additional benefits. Therefore, a novel ONS, containing ursolic acid, whey protein plus additional BCAAs and vitamin D, has been developed. In this study, we compare the effects of this novel ONS with a standard ONS on body composition, physical functioning, blood markers, and skeletal muscle gene and protein expression, in older adults with (or at risk of) undernutrition.

Methods

Study design

The ProMO-Study (PROtein supplementation in Malnourished Older adults) was a randomised, standard-care controlled, open-label trial conducted at Wageningen University, The Netherlands between July 2016 and August 2017. The primary aim of the study was to assess the effect of 12-weeks supplementation with a novel ONS on lean body mass. The study has been performed in accordance with the 1964 Declaration of Helsinki ethical standards and was approved by the medical and ethical committee of Wageningen University and registered at clinicaltrials.gov under NCT02683720.

Study population

Participants were recruited via dieticians, geriatric outpatient clinics of two hospitals (Rijnstate, Arnhem, The Netherlands and Gelderse Vallei, Ede, The Netherlands), the volunteer database of Wageningen University, and via advertisements in local and online media. Interested participants were, after obtaining informed consent, screened for eligibility during a home visit. We included participants aged 65 y and older, with a score below 12 on the Mini Nutritional Assessment Tool – short form (MNA-sf). Exclusion criteria were an expected life-expectancy of <12 months, mental state incompatible with proper study conduct, performing over 2 h / w of resistance exercise, unstable organ failure, impaired kidney function (estimated glomerular filtration rate (eGRF) <30 ml/min /1.73m^2, measured at baseline), allergy or sensitivity to milk proteins, chronic corticosteroid use, use of diabetes medication, change in medication use in the previous three months, use of antibiotics in the previous two months, use of oral nutritional supplementation in the previous three months, and use of >21 alcohol units per week.

Sample size

Sample size was calculated by using G-Power (G-Power Version 3.1.9.2. Kiel, Germany). To find a 1.0 kg difference in lean body mass between intervention and standard supplement after 12 weeks, with a standard deviation of 1.4 kg [16], we calculated that 64 participants provide a power of 80% at a two-sided alpha of 0.05. We assumed a drop-out rate of 20% and an after baseline exclusion for impaired kidney function of 1.5%, leading to a total sample size of 82 participants.

Randomisation

Participants were randomised by an independent researcher using a SAS-program (SAS Institute, Cary, NC) in permuted blocks of 4 participants, in a 1:1 ratio to intervention or standard supplement. The study was open-label: the supplements of the intervention and the standard supplement group differed in appearance and taste.

Study products

The novel supplement group consumed two portions of the novel ONS (Vital01, VitalNext B.V., Wageningen, The Netherlands) per day, each portion consisting of 63 grams of powder to be dissolved in a liquid of choice. The standard supplement group consumed two 200 ml bottles of Nutridrink (Nutridrink, Nutricia Advanced Medical Nutrition, Danone, Hoofddorp, The Netherlands) per day. The daily doses of the intervention and the standard supplement product contained equal amounts of energy, carbohydrates, fats and protein, but differed in the type of protein and the amount of BCAAs, vitamin D and UA (see **Table 1** for the nutritional content). Participants were advised to consume products after breakfast and lunch, but deviation from this advice was allowed to maximize compliance.

Table 1. The nutritional content of the study products per daily prescription (two portions).

	Standard supplement	Intervention
Energy (kcal)	600	586
Fat (g)	23	23
Carbohydrate (g)	74	65
Protein (g)	24	22
Of which casein (g)	24	11
Of which whey (g)	0	11
Free branched-chain amino acids (g)	0	7
Vitamin D3 (µg)	4.4	10.8
Ursolic acid (mg)	0	206

Study visits

Participants visited the Human Nutrition Research Unit of Wageningen University during three occasions (at week 0, 6 and 12). Participants arrived in the morning, after a light breakfast. Participants were free to choose their own light breakfast but were instructed to use exactly the same breakfast at the follow-up visits. At all three visits, body composition was assessed, and blood was collected. At all study visits, blood collection was always carried out before any other outcome measurements. At visit 1 and 3, additional measurements of physical function were performed, and vastus lateralis tissue was collected.

Body Composition

The primary outcome of this study was lean body mass, assessed via dual-energy x-ray absorptiometry (DXA, Lunar Prodigy Advance; GE Health Care, Madison, WI, USA). Phase angle and changes in intra- and extracellular water were measured by multi-frequency bioimpedance vector analysis (BIVA, SFB7, Impedimed Limited, Pinkeba, QLD, Australia). Height was measured to the nearest 0.1 cm, at baseline, using a stadiometer. Participants measured their body weight daily before breakfast at home, on commercial weighing scales (König HC-PS100N, NEDIS, 's Hertogenbosch, The Netherlands), to the nearest 0.1 kg. Bodyweight was also assessed during visits to the nearest 0.1 kg, with a calibrated weighing scale (ED-6-T; Berkel, Rotterdam, The Netherlands).

Strength

Isometric knee extension and knee flexion strength (in Newton) were measured by hand-held dynamometry (MicroFET2, HOGGAN Scientific LLC, Salt Lake City, UT, USA). Participants were seated on an examination table, with their knees flexed in a 90-degree angle. The examiner was seated against the wall for stability, provided standardised verbal encouragements and applied counterforce to the dynamometer. The dynamometer was placed just above the ankle joint, at the anterior side of the leg for extension measurements and the posterior side for flexion measurements. Participants were instructed to gradually increase their force within the first second after the standardised countdown ('3-2-1-GO'). After this second the participants provided maximum voluntary force for up to 4 seconds. For extension and flexion, participants started with one familiarisation trial, followed by six repetitions alternating between legs. Peak dominant and non-dominant flexion and extension force were used for analysis. Handgrip strength was assessed by hydraulic dynamometry (Jamar, Jackson, MI, USA) to the nearest kg while participants were seated on a chair without armrests, with their arms flexed in a 90-degree angle. Each hand was measured three times in alternating fashion. The highest readings of dominant and non-dominant handgrip strength were used for analysis.

Physical performance

Physical performance was assessed via the short physical performance battery (SPPB), which consist of (1) a 4-meter usual gait speed walking test, (2) a repeated chair rise test and (3) a balance test. On all three tests, a score between 0 and 4 was given following the original SPPB protocol [17]. The walking performance was assessed with the 400-meter walk test. Participants were instructed to walk 20 laps (20 m per lap, 400 m in total), at their usual gait speed, without using walking aids. Participants were allowed to take rest breaks if needed. Time (in seconds) to complete the 400 m was used for analysis.

Blood collection

At all three visits, phlebotomists drew two 3 ml serum tubes, one 5 ml EDTA-containing tube and one 3 ml Li-Heparin tube. The blood in serum tubes was allowed to clot at room temperature for 30 minutes. All tubes were centrifuged to separate plasma or serum. EDTA-plasma was divided over four 0.5 ml tubes, which were stored

at -80 degrees until analysis. Serum and Li-Heparin tubes were transported to an external laboratory (Hospital Gelderse Vallei, Ede, The Netherlands) for same-day analysis. Results of kidney function (estimated glomerular filtration rate, eGFR) were obtained on the same day to immediately exclude participants when eGFR dropped below <30 ml/min /1.73m^2. Levels of vitamin D (by Liquid chromatography-mass spectrometry), IGF-1 (by Luminescence-enhanced immuno-enzymatic assay) and albumin (by bromocersol purple method) were measured at the laboratory of Hospital Gelderse Vallei.

At the first and last visit, additional blood was collected in 9 ml citrate tubes, for immunological assessments. These tubes were transported to an external laboratory (Sanquin, Amsterdam, The Netherlands), where peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque separation, washed and counted. PBMC composition (T-cells, B-cells, monocytes and natural killer (NK) cells) was assessed by flow cytometry. PBMCs were stimulated (in triplicate) with a mix of Tetanus Toxoid, Tubulin PPD and Candida Albicans to trigger a memory reaction of T-cells. Production of interferon-gamma (IFNγ) and interleukin-13 (IL-13) was measured in supernatants by enzyme-linked immunosorbent assays.

Vastus lateralis tissue collection

Vastus lateralis tissue biopsies were taken under local anaesthesia, by using a Bergström needle, in a subgroup of *n*=36 participants who were willing to undergo a muscle biopsy and were not using anticoagulants. One part of the collected tissue was immediately rolled until dry, checked for quantity and type of tissue, and snap-frozen in liquid nitrogen for MicroArray analysis. A second part of the collected tissue was adhered to an object-glass with Tissue Tek (Sakura Tissue Tek, Alphen a/d Rijn, the Netherlands), and frozen in thawing isopentane, for Western Blot-analysis. Samples were frozen in -80 degrees until analysis. In *n*=21 participants, the tissue collection was successful both at baseline and after 12 weeks. We used these samples for the microarray analysis. In *n*=15 of these *n*=21 participants, enough tissue was collected to perform Western Blot analyses to assess protein expression.

RNA isolation and microarray processing

RNA was purified from muscle biopsies using TRIzol (Life Technologies, Bleiswijk, the Netherlands) followed by an additional round of purification with RNeasy Microkit columns (Qiagen, Venlo, the Netherlands). The RNA quality was assessed using RNA 6000 nanochips on the Agilent 2100 bioanalyzer (Agilent Technologies, Amstelveen, the Netherlands). Total RNA (100 ng) was labelled using an Affymetrix WT plus reagent kit and hybridized to whole-genome GeneChip Human Gene 2.1 ST arrays coding 25.088 genes and transcripts, (Life Technologies, Bleiswijk, the Netherlands). Sample labelling, hybridization to chips and image scanning was performed according to the manufacturer's instructions.

Microarray data analysis

Microarray analysis was performed using MADMAX pipeline for statistical analysis of microarray data [18]. Quality control was performed and all arrays met our criteria. For further analysis, a custom annotation was used based on reorganized oligonucleotide probes, which combines all individual probes for a gene [19]. Expression values were calculated using robust multichip average (RMA) method, which includes quantile normalisation [20]. Significant differences in expression were assessed using paired Intensity-Based Moderated T-statistic (IBMT [21]). Pathways analysis was performed by Gene Set Enrichment Analysis [22]. All microarray data are MIAME compliant and have been submitted to the Gene Expression Omnibus (identifier: GSE136395).

Western Blots

Proteins were isolated from the vastus lateralis tissue biopsies by homogenizing the biopsies in ice-cold RIPA Lysis and Extraction Buffer (25 mm Tris-HCl, pH 7.6, 150 mm NaCl, 1% Nonidet P-40, and 0.1% SDS; Thermo Fisher Scientific, Rockford, Il, USA) supplemented with protease and phosphatase inhibitors (Roche Diagnostics, Almere, The Netherlands). The protein lysates were quantified using the bicinchoninic acid assay (ThermoFisher) according to the manufacturer’s protocol. Protein lysates (12 µg of protein/well) were loaded and run on 8–16% Criterion gels (Bio-Rad, Veenendaal, The Netherlands), transferred onto PVDF membrane, blocked and probed with specific antibodies.

Membranes were incubated overnight with primary antibodies at 1:1000 dilutions for AMPK, p-AMPK, TFAM (respectively #2603, #2535, #7495, Cell Signaling Technology, Danvers, MA, USA), and PGC1α (#PA5-72948, ThermoFisher Scientific), and were followed by secondary antibody incubation with anti-rabbit IgG HRP from goat (#AP187P, Merck, Darmstadt, Germany) at 1:2500 dilution. Blocking and the incubation of primary and secondary antibodies were all performed in TBS, pH 7.5, 0.1% Tween 20 (TBS-T), and 5% (w/v) skimmed milk. Before and after the secondary antibody incubation, membranes were washed three times for 10 min each with TBS-T. Quantification was performed with the ChemiDoc MP system (Bio-Rad) and Clarity ECL substrate (Bio-Rad). GAPDH served as a loading control.

Dietary intake

Participants were instructed to keep their background diet unchanged during the study. Participants filled out a 2-day food record on the two days preceding the baseline visit, and on the same two days before the final visit. Additional interviews were performed by trained dietitians, using models to estimate portion size, with focus on food products high in energy, protein, calcium or vitamin D. Nutrient intake was calculated by using Dutch food composition database [23].

Physical activity

All participants were instructed to keep their level of physical activity equal during the study period. In a subset of n=27, physical activity was measured by accelerometry (ActiGraph GTX3, 2009, Pensacola, FL, USA). Participants wore the accelerometer on an elastic belt on the hip for 14 consecutive days during all waking hours, except during bathing or swimming. The accelerometers measured acceleration and deceleration in 3 spatial dimensions, which facilitates the calculation of vector magnitude. The epoch interval for the accelerometer was set at 60 seconds. ActiGraph version 6.13.3 (ActiGraph, Pensacola, FL USA) was used to initialise and synchronise the accelerometers. Participants who recorded less than 10 hours of wear time per day or had data for less than four days were excluded from the analyses (n=3). Non-wear time was defined as any period of consecutive zero-counts for a minimum of 20 minutes. Data were averaged and expressed in counts per minute (CPM).

Compliance

Compliance was measured via intake calendars and empty package count. Empty packages were collected during three home visits during the study period. At these visits, also new study products were delivered, and any issues regarding protocol adherence were evaluated. Participants rated products weekly on taste, smell and satiety on a 10 mm visual analogue scale. Participants were asked to report all side effects and to record every adverse event in a provided diary. Adverse events were further discussed with an independent physician, who also assessed the probability of a relation with the study products.

Statistical analysis

Data were analysed by intention to treat. Normality was assessed via visual inspection of QQ-plots. Baseline characteristics are presented as mean ± SD for normally distributed variables and median (p25-p75) for skewed variables. To test for changes over time, we used linear mixed models with time, treatment and time*treatment interaction as fixed effects and a random intercept for subjects. Correlated errors within repeated measurements were modelled with an autoregressive covariance structure. All models were adjusted for gender and height, and models for knee extension and flexion also for the observer, as this measure is especially prone to inter-examiner bias [24]. Additional adjustment for age and BMI was performed if it improved Akaike's Information Criterion. We used Bonferroni adjustment to account for multiple posthoc comparisons. Changes in protein expression and immunological responses were assessed via nonparametric tests (sign test for paired comparisons, and Wilcoxon-Mann-Whitney for comparisons between independent samples). All analyses were performed in SAS 9.4 (SAS Institute, Cary, NC), and graphs and figures were created using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA).

Results

Participants

A total of 82 participants enrolled in the trial between August 2016 and May 2017, with the final measurement performed in August 2017. A total of 12 participants did not complete the full study period, of which one dropped-out before baseline. Therefore, data of 81 participants were used for the modified intention to treat analyses (see **Figure 1** for flow chart). Participant characteristics were comparable between the study groups (**Table 2**). The range of MNA-sf scores (5-11) and the median MNA-sf scores (10) were the same in each group. In both groups, around 10% of participants had confirmed or severe sarcopenia, regarding the updated diagnosis recommendations by the European Working Group on Sarcopenia in Older People (EWGSOP2, [25])

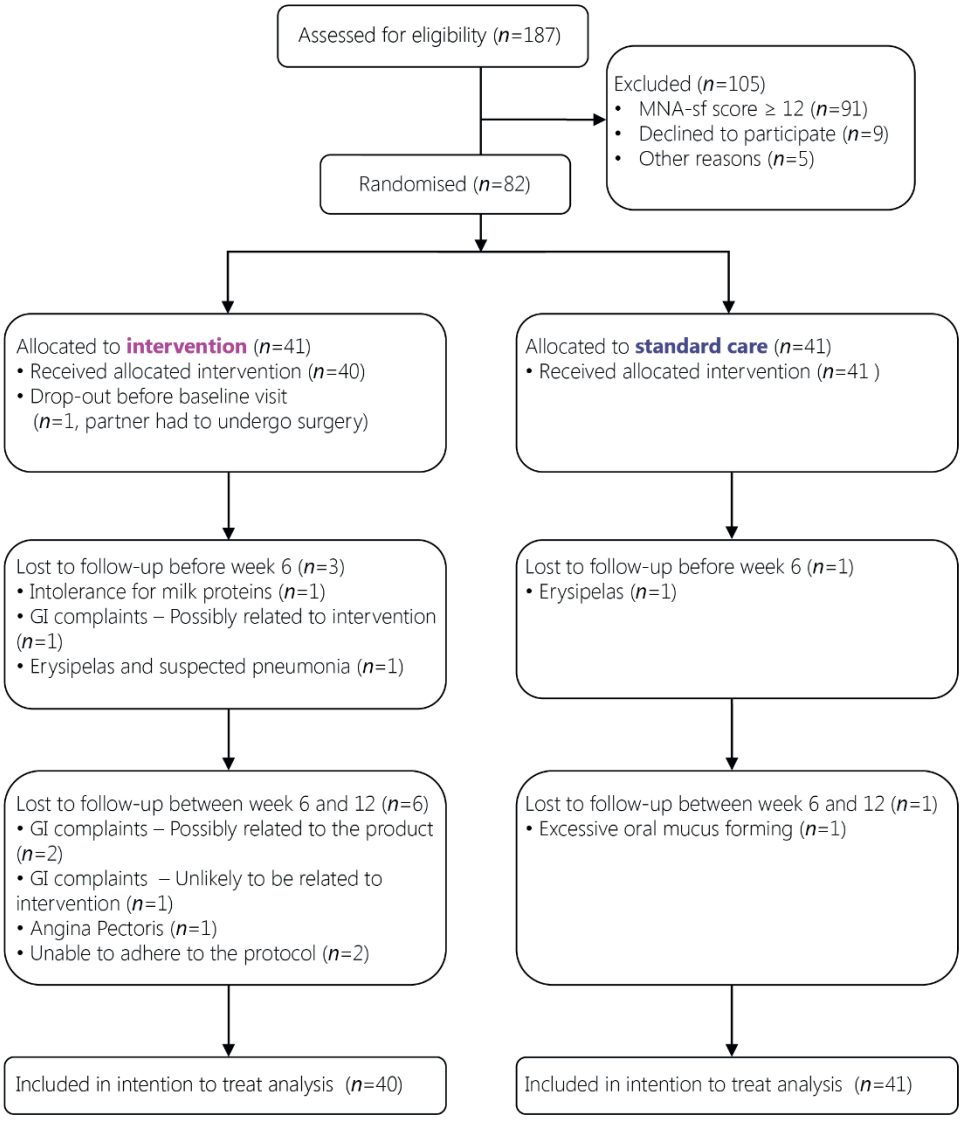

Figure 1 Flowchart of participants

Table 2. Baseline characteristics of the two study arms.

	Intervention (n=40)	Standard supplement (n=41)
Age, mean ± SD, y	74.5 ± 6.2	73.9 ± 6.8
Female, n (%)	22 (56%)	19 (44%)
Weight, mean ± SD, kg	61.6 ± 10.4	63.9 ± 10.8
Height, mean ± SD, cm	167 ± 9	171 ± 8
BMI, mean ± SD, kg/m^2	22 ± 3	22 ± 3
MNA-sf, median (IQR), score	10 (9-11)	10 (9-11)
Undernutrition (<9), n (%)	3 (7.3%)	6 (14.6%)
Risk of undernutrition (8-11), n (%)	37 (92.7%)	35 (85.4%)
EWGSOP2 sarcopenia classification		
No sarcopenia, n (%)	25 (61.0%)	22 (53.7%)
Sarcopenia probable, n (%)	12 (29.3%)	15 (36.6%)
Sarcopenia confirmed, n (%)	1 (2.4%)	2 (4.9%)
Sarcopenia severe, n (%)	3 (7.3%)	2 (4.9%)
Phase Angle, mean ± SD, °	4.8 ± 0.8	4.7 ± 0.7
Total lean mass, mean ± SD, kg	14.2 ± 6.5	13.1 ± 7.9
Appendicular lean mass, mean ± SD, kg	18.8 ± 4.2	20.6 ± 4.7
Protein intake, mean ± SD, g/kg/day	1.4 ± 0.4	1.3 ± 0.4
Energy intake, mean ± SD, kcal/day	2090 ± 452	2261 ± 548
25(OH)D_3, mean ± SD, nmol/l	84 ± 27	76 ± 32
Albumin, mean ± SD, g/l	38.8 ± 2.4	38.4 ± 2.4
Creatinine, , mean ± SD, µmol/l	75.5 ± 3.1	79.6 ± 3.7
IGF-1, mean ± SD, nmol/l	19.2 ± 6.6	16.0 ± 4.6
Walk time 4m, mean ± SD, s	4.2 ± 0.9	4.4 ± 1.4
Walk time 400m, mean ± SD, s	355 ± 76	362 ± 102
Dominant handgrip strength, mean ± SD, kg	22 ± 10	23 ± 10
Non-dominant handgrip strength, mean ± SD, kg	20 ± 10	21 ± 10
Dominant knee flexion strength, mean ± SD, N	193 ± 70	188 ± 70
Non-dominant knee flexion strength, mean ± SD, N	184 ± 69	185 ± 73
Dominant knee extension strength, mean ± SD, N	330 ± 112	325 ± 94
Non-dominant knee extension strength, mean ± SD, N	312 ± 99	311 ± 97
Chair rise test time, mean ± SD, s	11.5 ± 4.9	10.8 ± 3.2
SPPB Score, median (IQR)	11 (10-12)	12 (10-12)

MNA-sf, Mini Nutritional Assessment tool, short-form; EWGSOP2, European Working Group on Sarcopenia in Older People 2; IGF-1, Insulin-like Growth Factor 1; SPPB, Short Physical Performance Battery.

Body composition

Self-measured body weight significantly increased during the study period in the novel supplement group (Δ 1.6 ± 0.2 kg, P<.0001), and in the standard supplement group (Δ 1.8 ± 0.2 kg, P<.0001), without between-group differences (time*treatment effect P>0.05). The increase in lean mass was similar between the two treatment arms (Δ 0.28 ± 0.17 kg in the novel supplement group (P>0.05), and 0.23 ± 0.15 kg in the standard supplement group (P>0.05), time effect P=0.003, time*treatment effect P>0.05, **Figure 2**). Appendicular lean mass increased with 0.17 ± 0.07 kg in the full study population (P=0.07), with no significant time*treatment interaction. Fat mass increased in the novel supplement group with 1.1 ± 0.2 kg (P<.0001, Figure 2) and in the standard supplement group with 1.6 ± 0.2 kg (P<.0001, time*treatment effect P=0.045, Figure 2). Phase angle remained stable in the novel supplement group (Δ 0.02 ± 0.06 °; P>0.05) and increased in the standard supplement group (Δ 0.17 ± 0.05 °; P=0.027, time*treatment interaction P=0.07).

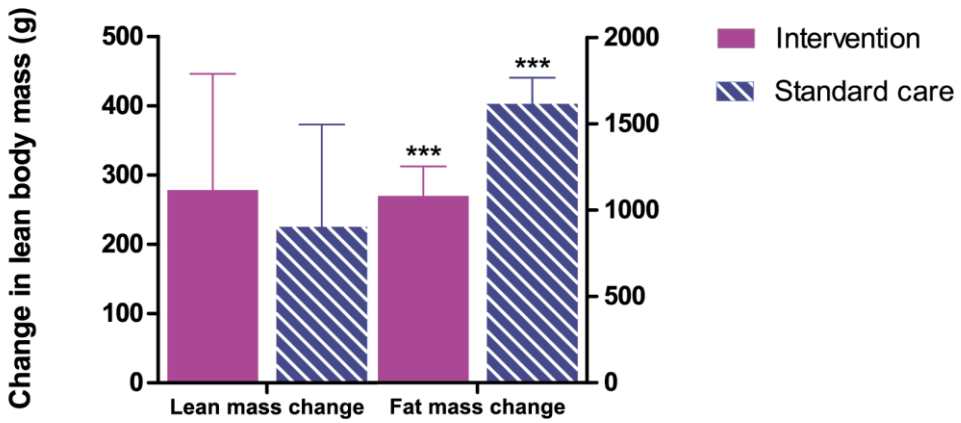

Figure 2 Change in lean mass and fat mass between baseline and end of follow-up (12 weeks) in the two treatment groups. ***, significant (P<.0001) within group change from baseline to week 12 on Bonferroni adjusted post hoc analyses from linear mixed models (n=81)

Physical function

The novel supplement group improved their performance on the 400 m walk test (Δ -7.3 ± 8.7 s, P>0.05), while the standard supplement group decreased their performance (Δ 17.6 ± 7.8 s, P>0.05; time*treatment effect P=0.038, **Figure 3**). The difference at the end of follow-up was 47 ± 23 s (P>0.05). Also on the 4-meter walk test, the novel supplement group improved their average time (Δ -0.4 ± 0.1 s, P=0.047), while the standard supplement group did not improve (Δ 0.0 ± 0.1 s, P>0.05, time*treatment interaction P=0.048, Figure 3). Non-dominant knee extension increased significantly within the standard supplement group (Δ 38 ± 10 N, P=0.003, time*treatment interaction P=0.058). No improvements were observed in other domains of physical function (**Table 3**). Activation of vastus lateralis, biceps femoris and rectus femoris as measured by surface electromyography did not differ within or between groups.

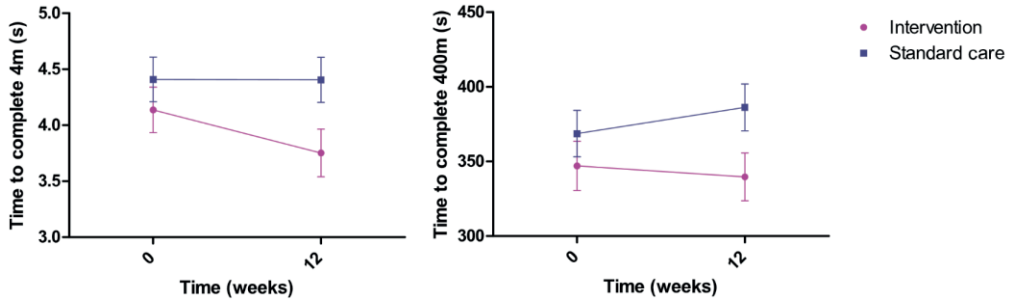

Figure 3 Change in time to complete 4 m (left, time*treatment effect P=0.047) and 400 m (right, time*treatment effect P=0.038, n=81)

Table 3. Changes in physical function in the two groups.

		Δ12 weeks				Time effect	Time* treatment interaction
		Intervention	P-value	Control	P-value[a]		
Walking performance	4 m [b]	-0.4 ± 0.1 s	0.047	0.0 ± 0.1 s	1.000	0.044	0.048
	400 m [c]	-7.4 ± 8.7 s	1.000	17.6 ± 7.8 s	0.172	0.386	0.038
Handgrip Strength[b]	Dom	0 ± 1 kg	1.000	0 ± 1 kg	1.000	0.974	0.948
	Non-dom	0 ± 1 kg	1.000	1 ± 1 kg	1.000	0.660	0.340
Knee extension[d]	Dom	-2 ± 14 N	1.000	26 ± 21 N	1.000	0.200	0.145
	Non-dom	8 ± 12 N	1.000	38 ± 10 N	0.003	0.005	0.058
Knee flexion[d]	Dom	12 ± 9 N	1.000	23 ± 8 N	0.036	0.006	0.351
	Non-dom	6 ± 10 N	1.000	15 ± 8 N	0.443	0.106	0.453
Chair rise test[c]		0.0 ± 0.5 s	1.000	-0.3 ± 0.4 s	1.000	0.543	0.634
SPPB score		0.1 ± 0.2 pt	1.000	0.3 ± 0.2 pt	0.523	0.169	0.355

Dom, dominant limb; non-dom, non-dominant limb; N, Newton; SPPB, short physical performance battery.

[a] P-values are from post hoc tests with Bonferroni adjustment of multiple testing

[b] Adjusted for gender and height

[c] Adjusted for gender, height and age

[d] Adjusted for gender, height, age and observer

Table 4. Top 10 enriched gene sets per treatment arm versus baseline, compared to the other arm

		Intervention (n=8)		**Standard supplement (n=13)**	
Top 10 enriched pathways in **novel supplement group**	Size	NES	FDR	NES	FDR
The citric acid TCA cycle and respiratory electron transport	149	2.93	$<10^{-4}$	-3.01	$<10^{-4}$
Respiratory electron transport ATP synthesis	106	2.91	$<10^{-4}$	-2.89	$<10^{-4}$
Mitochondrial translation initiation	84	2.87	$<10^{-4}$	-2.66	$<10^{-4}$
Mitochondrial translation elongation	84	2.86	$<10^{-4}$	-2.62	$<10^{-4}$
Respiratory electron transport	86	2.85	$<10^{-4}$	-2.84	$<10^{-4}$
Electron transport chain	88	2.85	$<10^{-4}$	-2.92	$<10^{-4}$
Mitochondrial translation	90	2.82	$<10^{-4}$	-2.65	$<10^{-4}$
Mitochondrial translation termination	84	2.80	$<10^{-4}$	-2.66	$<10^{-4}$
Mitochondrial protein import	60	2.79	$<10^{-4}$	-2.37	$<10^{-4}$
Oxidative phosphorylation	51	2.63	$<10^{-4}$	-2.58	$<10^{-4}$
		Intervention		**Standard supplement**	
Top 10 enriched pathways in **standard supplement group**	Size	NES	FDR	NES	FDR
Tyrobp causal network	60	1.11	0.58	3.09	$<10^{-4}$
Extracellular matrix organization	282	0.99	0.76	2.80	$<10^{-4}$
Beta1 integrin cell surface interactions	66	0.93	0.84	2.76	$<10^{-4}$
Neutrophil degranulation	462	0.93	0.83	2.71	$<10^{-4}$
Hsa04610 complement and coagulation	77	-0.97	0.87	2.64	$<10^{-4}$
Hsa05144 malaria	48	1.07	0.64	2.63	$<10^{-4}$
Complement and coagulation cascades	57	-1.17	0.68	2.59	$<10^{-4}$
Response to elevated platelet cytosolic ca2	131	-0.86	0.94	2.58	$<10^{-4}$
Platelet degranulation	126	-0.88	0.93	2.54	$<10^{-4}$
Interferon alpha beta signalling	65	-2.01	0.18	2.52	$<10^{-4}$

FDR, false discovery rate q-value; NES, normalised enrichment score

mRNA expression

The top 10 gene sets with the largest standardised enrichment scores per treatment are presented in **Table 4**. In the novel supplement group, all of these upregulated gene sets were related to oxidative phosphorylation or mitochondrial structures, while the same gene sets were significantly downregulated in the standard supplement group. The most enriched gene sets in the standard supplement group were related to blood coagulation and immune pathways.

Protein expression

Both treatments upregulated the expression of proteins that induce mitochondrial biogenesis (**Figure 4**). Activation of AMPK increased in both groups to a similar extent, but this difference was only significant in the novel supplement group (novel supplement group P=0.031, standard supplement group P=0.125 Figure 4B). TFAM and PGC-1α expressions were not differentially upregulated between the two groups (Figures 4C and 4D, P=0.603 and P=0.685 for between treatment differences in TFAM and PGC-1α fold changes, respectively).

Dietary intake and physical activity

Daily energy intake at baseline was 2090 ± 452 kcal in the novel supplement group and 2261 ± 548 kcal in the standard supplement group. Energy intake increased by 409 ± 95 kcal/d in the novel supplement group (p<0.001) and by 284 ± 87 kcal/d in the standard supplement group (p=0.01). Protein intake increased in both groups: in the novel supplement group from 83.4 ± 4.3 g/d to 102.8 ± 4.7 g/d (Δ 19.4 ± 4.4 g/d, P<0.001) and in the standard supplement group from 83.8 ± 4.3 g/d to 100.7 ± 4.3 g/d (Δ 16.9 ± 4.0 g/d, P<0.001).

Physical activity was equal between groups at baseline (Δ 138 ± 74 CPM, P=0.498) and end of follow-up (Δ 124 ± 88 CPM, P=1.000). No change in physical activity was observed in the intervention (Δ -33 ± 62 CPM, P=1.000) group and the standard supplement group (Δ -19 ± 47 CPM, P=1.000).

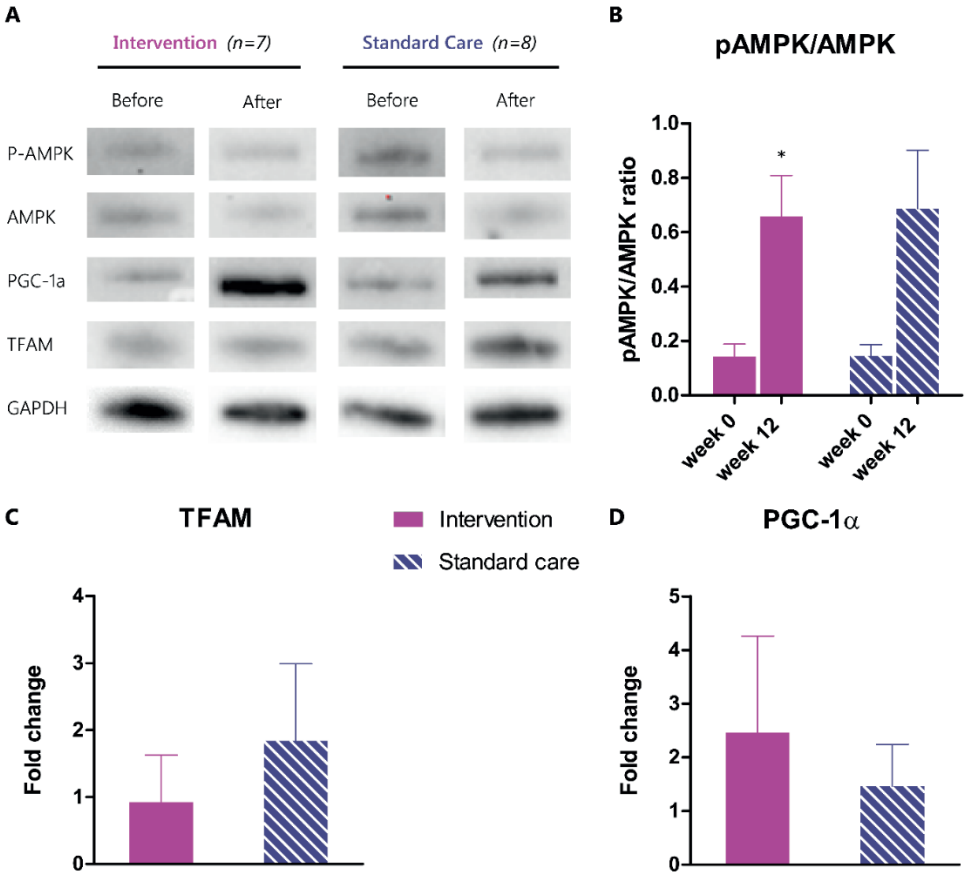

Figure 4 Effects of the two treatments on expression levels of proteins involved in mitochondrial pathways (n=15). Representative captures from western blots (A), of which quantification (mean plus standard error bar) is presented in B, C and D: pAMPK/AMPK ratios per treatment arm and per measurement time (B), fold change between week 0 and 12 in arbitrary units of TFAM (C) and PGC-1α (D) expression. All protein densities were normalised against GAPDH expression. *, significant (P<.05) within-group change from baseline to week 12

Blood markers

Mean baseline vitamin D levels were 83.8 ± 3.3 nmol/l in the novel supplement group and 76.2 ± 3.9 nmol/l in the standard supplement group (Δ 5.7 ± 5.9 nmol/l, P=1.000). Both groups showed similar, highly significant increases in vitamin D status throughout the study period. Status levels increased on average with 13.3 ± 3.0 nmol/l (P<0.001) in the novel supplement group, and with 12.7 ± 2.7 nmol/l (P<0.001) in the standard supplement group. IGF-1 levels in the novel supplement group were similar between groups at baseline (19.2 ± 0.9 nmol/l vs 16.1 ± 0.7 nmol/l, Δ 3.0 ± 1.3 nmol/l, P>0.05). IGF-1 levels decreased in the novel supplement group (Δ -0.9 ± 0.6 nmol/l, P>0.05), while they increased in the standard supplement group (Δ 1.3 ± 0.6 nmol/l, P>0.05), resulting in a significant time*treatment effect (P=0.025).

Blood creatinine levels decreased from 75.5 ± 3.1 µmol/l to 72.3 ± 3.1 µmol/l in the novel supplement group, while the standard supplement group showed a minor increase from 79.6 ± 3.7 µmol/l to 80.5 ± 3.7 µmol/l (time*treatment interaction P=0.004). Albumin decreased in the standard supplement group with 1.0 ± 0.4 g/l (P=0.08) and in the novel supplement group with 2.2 ± 0.4 g/l (P<.0001).

Immune function

Experiments on PBMCs were performed in *n*= 69 participants who completed the before and after blood collection. Composition of the PBMCs changed in both groups. In the standard supplement group, an increase in %T-cells (61 ± 11% to 63 ± 10%, P<0.05) was observed, at the expense of a decrease in %NK-cells (17 ± 8% to 15 ± 7%, P<0.001). The novel supplement group showed an increase in %T-cells (56 ± 12% to 61 ± 12%, P<0.01) and %B-cells (5.9 ± 2.3% to 6.4 ± 2.6%, P=0.064), and a decrease in %monocytes (16 ± 8% to 12 ± 5%, P<0.01) and %NK-cells (17 ± 9% to 15 ± 8%, P<0.01). In both groups, there were no differences in before-stimulation levels of IL-13 and IFNγ at baseline compared to end-of follow-up. Stimulation of PBMCs with memory recall mixes significantly increased levels of IL-13 and IFNγ in both groups at both time points (P<.0001 for all comparisons). Before and after stimulation levels of IL-13 and IFNγ did not differ between groups on both time points.

Tolerance

Median compliance was high (95%) and did not differ between treatment arms. The taste of the standard supplement product was rated better on a 100 mm VAS scale, than that of the intervention product at the start of the trial (standard supplement: 57 ± 3.6 mm vs intervention: 40 ± 3.6 mm, P=0.001). This difference attenuated during the trial, resulting in a tendency to a higher rating for the standard supplement product at the end of follow-up (standard supplement: 60 ± 4.7 mm vs intervention: 49 ± 4.7 mm, P=0.085).

During follow-up, a total of 4 serious adverse events occurred, which were not related to treatment nor to study procedures. All serious adverse events (angina pectoris, erysipelas with suspected pneumonia, transient ischemic attack, volvulus) occurred in the novel supplement group, and the first two led to discontinuation of the participant. Adverse events were reported by 31 (77.5%) participants in the intervention arm and 28 (68.3%) participants in the standard supplement group. A total of 55 adverse events were classified as gastrointestinal complaints. Upper abdominal complaints, such as belching, heartburn and dyspepsia, were reported more often by participants in the standard supplement group (n=10, leading to drop-out in n=1) than in the novel supplement group (n=5). Side-effects related to defecation, mainly reported as diarrhoea, were observed more often in participants in the novel supplement group (n=21) than in the standard supplement group (n=7). In the novel supplement group, these complaints led to study discontinuation in 5 participants. The relation to the study product was evaluated by an independent physician and considered ‘definite’ in n=1 (intolerance for milk proteins), ‘possible’ in n=3 and ‘unlikely’ in n=1 of the participants who dropped out.

Discussion

In this study, twelve weeks of supplementation with a novel ONS containing a mixture of casein and whey protein, additional free branched-chain amino acids, additional vitamin D, and ursolic acid, did not result in a greater increase in lean mass than standard supplement did. However, the novel ONS did lead to an improved gait speed during short and long distance. On a cellular level, the novel ONS upregulated pathways that are associated with mitochondrial biogenesis and oxidative phosphorylation.

Total lean mass did increase in the total study population, with 0.25 kg (of which 0.17 kg was appendicular lean mass, ALM), but this increase was not different between the two treatments. The 0.17 kg increase in ALM was in line with findings from the PROVIDE study, which found a 0.17 kg higher ALM after 13 weeks of supplementation with a whey protein-based ONS compared to non-protein containing control in older adults with sarcopenia [14]. In our study, the standard supplement led to a considerably greater increase in fat mass than the intervention product (1.6 vs 1.1 kg). This difference in fat mass gain might be due to anti-obesity effects of UA that have been reported in multiple animal studies [26] and in one human trial [27]. It is unlikely that the different types of proteins in the supplements caused this difference [28].

Participants in the novel supplement group improved their walking performance on the 4m and the 400m walk test, while participants in the standard supplement group showed a stable (4m walk test) or even decreased walking performance (400m walk test). The possible improvements in oxidative phosphorylation and the upregulated mitochondrial biogenesis in the novel supplement group, which were suggested by our mRNA expression analyses, form our most likely explanation for this finding. The improvements in gait speed that we observed are clinically relevant [29, 30], and might lower the chance of falls, hospitalisation and mortality [31, 32].

The most likely compounds in the novel product to be responsible for the effects on gait speed via improved oxidative metabolism are the free BCAAs and UA. BCAAs have shown to enhance mitochondrial biogenesis and endurance capacity in mouse models, which is mainly explained by an upregulation of mTOR [33, 34]. In our study, we did not observe upregulation of the mTOR pathway in either treatment arm.

However, BCAAs also upregulate PGC-1α and SIRT-1 [33]. We also observed an upregulation of protein expression of PGC-1α, but we were not able to detect SIRT-1 proteins with western blots. Moreover, BCAAs are precursors of acetyl-CoA and succinyl-CoA, which are components of the TCA-cycle, and might, therefore, lead to upregulations of the TCA-cycle gene set.

Where our results are partly similar to those found with BCAAs in mouse models, our results are even more in line with results from studies using UA in mouse models. Chen and colleagues showed that UA improved mitochondrial biogenesis via upregulation of activated AMPK, TFAM and PGC-1α in myotubes, and improved endurance in mice [13]. In the subsample of n=15 participants from which we successfully assessed protein expression via western blots, we found results that are in line with the findings of Chen et al. We observed increased activation of AMPK in both treatment arms, and signs of increased expression of PGC-1α, which was especially pronounced in the novel supplement group. TFAM was also upregulated in both treatment arms, but in this case, the expression was increased to a greater extent in the standard supplement group. Within-group upregulations of TFAM and PGC-1α were however not significant due to large variation. Although the protein expression analyses suggest that mitochondrial pathways are upregulated in both treatments, we only found evidence for an enrichment of gene sets related to oxidative metabolism and mitochondrial biogenesis in the novel supplement group. Our study design limits our ability to confidentially determine the compound that is responsible for the observed effects. However, our results on endurance and mitochondrial protein expression are strikingly similar to the results found with isolated UA on muscle fibres and in mouse models by Chen et al. Therefore, we do urge the need of highly controlled trials that investigate the role of isolated UA on endurance capacity and mitochondrial functioning in older adults, to further explore the potential therapeutic role of UA in this population.

In line with the comparable improvements in lean body mass in the two groups, we did not observe between treatment differences in muscle strength. Handgrip strength, total SPPB score and chair rise test did not show any sign of a change in either group. Extension and flexion of the legs increased in both groups, but only the extension of the non-dominant leg showed between-treatment differences, with a trend towards a beneficial effect of the standard supplement over the novel supplement group. However, the increase in non-dominant flexion in the standard supplement group failed to reach a significant time*treatment effect and did not

translate in improved performance on chair rise test or the two walking tests. This suggests that in the absence of an exercise intervention, treatment with the novel product or with standard supplement does not lead to different results on lean body mass or muscle strength.

We observed a time*treatment effect on IGF-1 levels, with an increase of 1.3 nmol/l in the standard supplement group, and a decrease of -0.9 nmol/l in the novel supplement group. As IGF-1 levels were 3.0 nmol/l higher in the novel supplement group at baseline compared to the standard supplement group, this observed effect could be partly explained by regression to the mean. Moreover, there is evidence showing that only casein protein, and not whey, increases IGF-1 levels [35]. These different effects of whey and casein on IGF-1 levels possibly explain why the standard supplement product, which contained 24 g of casein per daily dose, resulted in increased IGF-1 levels, while the intervention product, which contained 11 g of casein and 11 g of whey per daily dose, did not increase IGF-1 levels. Moreover, although UA stimulated IGF-1 signalling in skeletal muscle tissue, it did not increase plasma IGF-1 levels in mouse models [12]. The exact effects of plasma IGF-1, which is primarily produced hepatically under the influence of growth hormone, on lean body mass in older adults are unclear [36, 37]. Also, in our study, the between-group differences in plasma IGF-1 levels did not translate into differences in lean body mass.

In both groups, participants showed high compliance to the study products. Thereby, the standard supplement and the intervention product successfully increased protein and energy intake. Participants in this study already had a high mean protein intake (1.4 g/kg/day), energy intake (2177 kcal/day) and vitamin D status (80 nmol/l) at baseline. These high intake levels might be explained by increased intake or over-reporting, as a result of increased awareness towards food intake. A cause for this could be the nutritional assessment (by MNA-sf) performed at the screening visit, before the days on which the baseline food records were completed, or the fact that some participants were already seeing a dietitian, and dietary advice other than ONS may already have been implemented (for instance consuming more dairy). Although the daily vitamin D intake from ONS was more than twice as high in the novel supplement group (10.8 µg) than that in the standard supplement group (4.4 µg), both groups showed a similar increase in vitamin D status. This can be explained by the high baseline vitamin D levels, variations in vitamin D intake from the background diet and the allowed use of vitamin D supplementation during the study. Despite the compliance to the nutritional therapy and the improvements in body weight, serum

albumin levels decreased in both treatment arms. This observation supports the growing consensus that albumin levels are not suitable as a marker for nutritional status, as many other factors influence serum albumin levels [38]. Results of the stimulation of PBMCs with memory recall mixes did not suggest any changes in immune function on both groups. This suggests that 12-week treatment with medical nutrition is not improving immune function. However, we cannot rule out the possibility that this null-effect is due to large variations in immune responses in this heterogenic study population.

This study has several strengths. This study is one of the larger randomised controlled trials with ONS run in community-dwelling participants with (risk of) undernutrition. Trials with ONS in undernourished older adults are scarce, are often lacking assessment of important outcomes such as physical functioning and body composition, and often do not have a suitable comparator therapy [39]. We compared the novel supplement to standard supplement, which allows examination of its real added value. The broad range of measured outcomes provides an extensive picture of the effects of the intervention product and the standard supplement product. Notably, the inclusion of muscle mRNA and protein expression analyses provided crucial information on underlying mechanisms. We observed higher median compliance (95%) compared to other studies that investigated ONS in community settings (81%) [40]. Moreover, while we anticipated a drop-out rate of 20%, only 13.5% of participants did not complete the study due to a variety of reasons. In the standard supplement group, drop-out rates were low, with only 2 of 41 participants dropping out. The drop-out rate was higher in the novel supplement group, partly due to gastrointestinal complaints possibly related to the study product in 4 of 40 participants. One of these participants appeared to be intolerant to milk protein. For the other three, we did not find an explanation for their reaction to the product, and an independent physician evaluated these complaints as being possibly related to the study product.

This study also faced some limitations. First, due to the different appearance of the study products, blinding was not possible. However, participants did not know which product was the intervention product and which was the control product. In this way, all participants were under the impression that they received the product of interest, which prevented a placebo effect in the novel supplement group. Besides, several objective outcomes (such as DXA and mRNA expression) are not prone to caveats of a non-blinded design. For rated outcomes, examiners were extensively trained on

following standardised protocols to avoid influencing results. Second, the multiple differences between study products make it impossible to identify with certainty which compound is responsible for the found effects. Third, we advised participants to consume the products after breakfast and lunch, but they were free to follow this advice or to distribute the supplements over the day in their preferred way. As a result, some participants may not have reached the anabolic threshold during every meal, but this did contribute to high compliance.

In conclusion, we showed that 12-week supplementation with a novel ONS improved walking performance in older adults with (or at risk of) undernutrition, possibly via improvements in mitochondrial mechanisms. Compared to standard supplement, the novel supplement performed equally well on other measured domains of physical function and body composition.

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Chapter 5

Vitamin B-6 intake is related to physical performance in European older adults - the NU-AGE study

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Submitted

Abstract

Background Older adults often have a decreased quality of life due to attenuated physical performance. Maintenance of high physical performance during ageing might be supported by an adequate dietary intake of niacin, vitamin B-6, B-12, and folate, as these B-vitamins are involved in multiple processes related to muscle functioning. However, not much is known about the association between dietary intake of these B-vitamins and physical performance.

Objective To investigate the association between dietary intake of niacin, vitamin B-6, B-12, and folate and physical performance in older adults, and explore mediation by niacin status and homocysteine concentrations.

Methods We used baseline data of the NU-AGE trial, which included *n*=1249 healthy older adults (aged 65-79 y) with complete data on dietary intake measured with 7-day food records and questionnaires on vitamin supplement use, and physical performance measured with the short physical performance battery and handgrip dynamometry. Associations were assessed by adjusted linear mixed models, and mediation analysis was performed with the Preacher and Hayes method.

Results Intake of vitamin B-6 was related to lower chair rise test time (β -0.033 ± 0.016 s (log), P=0.002). Vitamin B-6 intake was also significantly associated with handgrip strength, but for this association, a significant interaction effect between vitamin B-6 intake and physical activity level was found. In participants with the lowest level of physical activity, higher intake of vitamin B-6 tended to be associated with greater handgrip strength (β 1.5 ± 0.8 kg, P=0.051), while in participants in the highest quartile of physical activity higher intake was associated with lower handgrip strength (β -1.4 ± 0.7 kg, P=0.041). No evidence was found for an association between intake of niacin, vitamin B-12 or folate and physical performance, or for mediation by niacin status or homocysteine concentrations.

Conclusion Vitamin B-6 intake was associated with better chair rise test in a population of European healthy older adults, and with greater handgrip strength in participants with low physical activity only. Homocysteine concentrations did not mediate these associations.

Introduction

Life expectancy has been steadily increasing in the past 200 years [1]. However, the extra years of life are often lived in poor health. To age more healthily, older adults need to maintain a high level of physical performance in daily life, as this is related to increased independence and a higher quality of life [2]. Furthermore, high physical performance reduces chances of hospitalisation [3], cognitive decline [4] and mortality [5]. B-vitamins have been suggested to protect against age-related physical decline [6]. However, the exact role of B-vitamin intake in relation to physical performance in older adults is unknown, and evidence for associations is scarce.

B-vitamins play an important role in at least three mechanisms related to physical performance. Firstly, intake of vitamin B-6, B-12 and folate reduces homocysteine concentrations [7]. High concentrations of homocysteine induce inflammation and decrease neurological functioning, which might lead to decreased physical performance [8]. This is in line with multiple studies that have shown that homocysteine concentrations are inversely associated with quadriceps strength [9], chair rise test performance [10], and gait speed [10-12]. Secondly, niacin, vitamin B-6, B-12 and folate contribute to the functioning of mitochondria [13]. Mitochondrial functioning is impaired by elevated homocysteine concentrations [14]. Thus, lowering intracellular homocysteine concentrations by adequate vitamin B-6, B-12 and folate intakes may improve mitochondrial functioning, which can benefit physical performance [15]. In addition, dietary intake of niacin (and it precursor tryptophan) can lead to increased levels of nicotinamide adenine dinucleotide (NAD) [16]. Increasing NAD concentrations by pharmacological agents has shown to be an effective intervention to improve mitochondrial functioning in humans [17]. In mice, long-term administration of an NAD precursor reduced age-related physiological decline [18]. Thirdly, B-vitamins may preserve muscle quality via their antioxidant properties [19] and by improving neuromuscular functioning [6]. Taken together, B-vitamins may contribute to physical performance via suppressing homocysteine concentrations, improving mitochondrial functioning, and preserving muscle quality.

Despite these indications of an important role of B-vitamin intake in relation to physical performance, only two observational studies have investigated this association before, within the same cohort. These studies showed that adequate intake of vitamin B-6, B-12, and folate led to lower risk of impaired mobility [20] and frailty [21]. In these two studies, dietary intake was assessed via the dietary history method, and level of physical performance was self-reported. In this paper, we investigate the association between objectively measured physical performance as measured by handgrip strength and walking speed, with dietary intake as measured by detailed 7-day food records, in a large European cohort of older adults. In addition, we explored potential mediation by homocysteine concentrations and niacin status.

Methods

Study design

We used baseline data of the NU-AGE study for this analysis. The NU-AGE study is a dietary intervention trial that was performed in five centres in different European countries (France, Italy, the Netherlands, Poland and the United Kingdom). It is registered at clinicaltrials.gov under NCT01754012. The study design and participant recruitment have been described in detail before [22, 23]. In short, 2668 apparently healthy, community-dwelling older adults (aged 65-79 y) were recruited for the trial, of which 1296 eligible participants were included. For this study, we used data of 1249 participants who completed the baseline dietary intake assessment.

Dietary intake

Dietary intake was assessed through 7-day food records. Before the measurement period, participants were trained in describing foods, portion sizes and preparation methods. Trained dietitians or research nutritionists reviewed the level of detail of the completed food records during a home visit (in the Netherlands and UK) or at the research centre (Poland, Italy and France). Food records were coded according to standardised coding procedures. Nutrient content was calculated by using local food composition tables (NEVO 2011 in the Netherlands [24], McCance and Widdowson in the UK [25], INRAN and BDA in Italy [26, 27], NFNI in Poland [28] and CIQUAL French food composition table in France [29]). Participants from the French centre were excluded for vitamin B-12 analyses, due to unreliable intake data for this vitamin. Supplementary vitamin B intake was assessed by means of an additional questionnaire, and participants using vitamin B-containing supplements were identified and excluded from a sensitivity analysis. Niacin equivalents were calculated as following: niacin (mg) + (tryptophan(mg)/60). Tryptophan intake was estimated from total protein intake by assuming an average abundancy of 1.1% [30].

Physical performance

Physical performance was measured at all centres by the short physical performance battery (SPPB) and handgrip strength. The SPPB was performed following the protocol described by Guralnik et al. [31] and consisted of a 2.44 m usual gait speed test, a balance test in three-foot positions of increasing difficulty, and a five-time chair rise test. Handgrip strength was measured by dynamometry (Scandidact Smedley's Hand Dynamometer) to the nearest 0.1 kg, in standing position with the

arm flexed at 90 degrees. For each hand, the maximum value of three repetitions was used for analysis. The measurements of physical performance were standardised over the five centres. The standardisation was done by joint training sessions in Warsaw and Wageningen, where representatives from all five centres were present. Physical activity was expressed in Physical Activity Scale for Elderly (PASE) questionnaire-based PASE-scores [32]. These questionnaires were self-administered and checked by a researcher.

Biochemical analyses

Urine samples were collected during the seventh day of dietary intake assessment, which is one day prior to physical performance assessment. Participants were instructed to discard the first urine of the day and to collect all following urine including the first urine of the second day, in standard containers which contained 2.7 ml of 1% sodium azide solution. Total urine volume was recorded, and urine was aliquoted in cryovials and stored at -80 degrees C until analysis. Urinary N1-Methylnicotinamide (NMN) and N-methyl-2-pyridone-carboxamide (2-Pyr) concentrations were measured at University Medical Centrum Groningen, the Netherlands, by validated liquid chromatography (Luna HILIC column, Phenomenex) isotope dilution tandem mass spectrometry method (Quattro Premier, Waters), as described previously [33]. The sum of the two metabolite concentrations (µmol/L) was multiplied by total 24-h urine volume (L/day) to obtain 24-h urinary niacin metabolite excretion, which is considered a biomarker of niacin status[34].

Plasma homocysteine concentrations were assessed by enzymatic method (Olympus AU400 chemistry analyser by Beckman) in the Nigrisoli hospital in Bologna, Italy, as described before [35].

Statistical analysis

For descriptive purposes, energy-adjusted tertiles of the four vitamins (B3, B-6, B-12 and folate) were created by using the residual method [36]. Descriptive statistics are presented as means (SD) for normally distributed values, and medians (25th – 75th percentile) for skewed variables. Skewed outcome variables (walk time and chair rise test time) were log-transformed. All analyses were performed in SAS 9.4 (SAS Institute Inc.).

Associations between intake of B-vitamins and measures of physical performance were assessed via mixed linear models. Continuous, unadjusted measures of the B-

vitamins were used as exposure, and energy adjustment was performed via adding energy intake to the model. Possible effect-modification by physical activity level was tested by adding the interaction term exposure*physical activity to the model. The interaction model was used when the interaction effect was significant, or when adding the interaction term changed the direction of the beta.

Three models of increasing complexity were built to adjust for confounding factors. The first model only adjusted for age and sex. The second model additionally adjusted for energy intake, drinking status (non, light or heavy), smoking status (never, former or current), education level (low, medium or high) and physical activity level, and the third model additionally adjusted for protein intake and study centre. Adjustment for study centre was performed by including study centre as a random factor in a random intercept model.

Additional analyses were done to test for a possible mediating effect of niacin metabolites in the niacin – physical function relationship and for homocysteine concentrations in the vitamin B-6, B-12, folate – physical performance relationship. We calculated a normalised combined vitamin score for vitamin B-6, B-12 and folate, by dividing all values by the mean intake of the specific vitamin and summing up those values. In other words, if a participant has an intake around the mean for vitamin B-6, B-12 and folate, the combined score for this participant would be ~3. We followed the mediation analysis method described by Preacher and Hayes [37] and concluded no evidence for mediation when the potential mediator was not associated with either exposure or outcome. Associations between mediator and exposure or outcome were performed by using the fully adjusted models.

We performed two sensitivity analyses. The first sensitivity analysis was performed to assess the influence of possible under or overreporters of energy intake. For this analysis, we excluded participants with a daily average energy intake outside the 500 - 4000 kcal range, and participants with an energy intake to basal metabolic rate (estimated by Schofield's equation [38]) ratio outside the 0.8 – 2.66 range. In the newly created dataset, we reran the programs of the second and third models and compared outputs. The second sensitivity analysis excluded participants using vitamin B-containing supplements.

Results

Participants (*n*=1249) had a mean age of 71 ± 4 years, and 56% were female (**Table 1**). The study population had a high level of physical functioning, with a median SPPB score of 12. Measures of physical performance were similar over the five centres, except for high handgrip strength in participants from the UK, even despite the higher female proportion in this centre. The UK participants also showed higher scores on the PASE-questionnaire and had the highest intake levels of niacin, vitamin B-6 and B-12. Participants from France had the highest daily average energy and protein intake, but the lowest BMI. On the other hand, participants from Italy had the lowest average daily intake of energy, protein and vitamin B-12, and the lowest score on the PASE-questionnaire. In the Netherlands, participants had the lowest intake levels of three out of four B-vitamins: niacin, vitamin B-6 and folate.

Characteristics of the participants per energy-adjusted tertiles of niacin, vitamin B-6, folate or B-12 intake are presented in **Table 2**. With the exception of folate intake, the gender distribution was similar in all three tertiles. For folate intake, a higher proportion of males was observed in the first tertile compared to the other tertiles. Participants with higher folate intake were more likely to have a lower body weight and handgrip strength, whereas participants with higher intake of niacin, vitamin B-6 and B-12 were more likely to have higher body weight and handgrip strength. Other measures of physical performance were similar in the different tertiles of all the vitamins. Participants with lower intakes of vitamin B-6, B-12 and folate were more likely to have fewer years of education. Low folate-consumers were more often heavy drinkers (≥14 units per week), while low-B-12 consumers were more often non-drinkers. Participants with higher intake of any of the four vitamins consumed more protein and more of the other three B-vitamins. Significant correlations were observed for the six pairs of B-vitamins, ranging from r=0.22 for the correlation between intake of niacin and vitamin B-12 to r=0.61 for the correlation between intake of vitamin B-6 and folate. Participants with a high intake of folate or vitamin B-12 were more likely to have lower homocysteine concentrations.

Table 1. Baseline characteristics of the total study sample and per study centre. Data presented as mean (SD) unless stated otherwise.

	Total (n=1249)	Italy (n=273)	United Kingdom (n=272)	The Netherlands (n=252)	Poland (n=251)	France (n=201)
Gender (% female)	56	52	64	56	57	50
Age (y)	70.9 (4.0)	71.7 (3.9)	70.1 (4.0)	71.0 (4.0)	71.4 (3.8)	71.2 (3.9)
Height (cm)	166 (9)	164 (10)	166 (9)	169 (8)	164 (9)	166 (9)
Weight (kg)	73.6 (13.4)	73.0 (12.6)	74.0 (13.7)	74.6 (13.2)	75.3 (14.2)	70.1 (12.6)
BMI (kg/m^2)	26.7 (4.0)	27.2 (3.9)	26.8 (4.1)	26.0 (3.6)	28.0 (4.2)	25.4 (3.5)
Smoker (%never/former/current)	53/42/5	44/49/7	60/38/2	51/46/3	49/44/7	66/31/3
Drinker (%non/ light/ heavy)[b]	17/71/11	31/53/16	10/82/9	13/72/15	21/55/24	10/71/18
Education (low/medium/high)[c]	13/52/35	39/39/22	1/83/16	13/56/31	1/28/71	11/53/36
PASE-score	133 (55)	113 (50)	149 (53)	137 (53)	131 (62)	133 (52)
Homocysteine (µmol/L)	12.6 (4.3)	14.5 (4.2)	12.8 (3.8)	11.1 (3.0)	12.5 (5.2)	11.9 (4.2)
Niacin intake (mg/d)[a]	17.0 (13.9-20.7)	17.2 (13.7-20.9)	18.4 (15.6-22.2)	15.7 (13.3-18.6)	17.0 (13.4-21.3)	16.5 (13.7-20.0)
Vitamin B-6 intake (mg/d)	1.8 (0.6)	1.6 (0.5)	2.0 (0.6)	1.6 (0.5)	2.0 (0.7)	1.7 (0.4)
Folate intake (ug/d)	290 (97)	271 (106)	309 (96)	255 (71)	297 (98)	323 (93)
Vitamin B-12 intake (ug/d)[a]	4.3 (3.0-6.3)	2.7 (1.9-4.1)	5.9 (4.5-7.8)	4.3 (3.4-5.6)	4.1 (2.9-6.3)	*N.A.*
Protein intake (g/d)	75 (18)	67 (14)	77 (16)	76 (16)	77 (22)	81 (17)
Energy intake (kcal/d)	1864 (445)	1711 (381)	1895 (382)	1908 (411)	1822 (512)	2026 (484)
B-supplement users (%)	19	15	20	26	21	11
Dominant hand grip strength (kg)	31.5 (9.5)	30.9 (9.7)	34.5 (9.0)	30.5 (9.2)	30.3 (9.8)	31.0 (8.8)
Total SPPB score[a]	12 (11-12)	12 (11-12)	12 (11-12)	12 (11-12)	12 (11-12)	12 (11-12)
Repeated chair rise time (s)	10.3 (2.8)	10.3 (2.9)	10.6 (2.8)	10.0 (2.7)	10.2 (2.7)	10.3 (2.6)
Time to walk 2.44 meters (s)	2.4 (0.6)	2.3 (0.6)	2.3 (0.6)	2.5 (0.5)	2.5 (0.6)	2.6 (0.5)

[a]median (IQR) [b] light-drinker, 1 to 14 units per week; heavy-drinker, 14 or more drinks per week. [c] low, 0-8 years; medium, 9-13 years, high, 14 or more years.

BMI, body mass index; PASE-score, score on the Physical Activity Scale for the Elderly; SD, standard deviation; SPPB, short physical performance battery.

Table 3 presents the associations between intake of the four B-vitamins and measures of physical performance. Intake of vitamin B-6 was related to improved chair rise test time (β -0.033 ± 0.016 s (log), P=0.002), translating to a 3.2% lower chair rise time per mg higher vitamin B-6 intake. Niacin and vitamin B-6 intakes were significantly and positively associated with handgrip strength. However, for these associations, there was a significant interaction between vitamin intake and physical activity level. In participants with the lowest level of physical activity, intake of vitamin B-6 tended to be positively associated with handgrip strength (Table 4, β 1.5 ± 0.8 kg, P=0.051), while the associations were negative in participants with the highest level of physical activity (β -1.4 ± 0.7 kg, P=0.041, interaction effect P=0.017). There were no significant associations between niacin intake and handgrip strength at any physical activity level. Participants in the highest physical activity quartile had a higher handgrip strength than those in the lowest physical activity quartile (Δ4.4 ± 0.7 kg, P<0.0001). They also had a higher intake level of total energy (Δ170 ± 37 kcal, P<0.001), protein (Δ6 ± 1 g, P<0.0001), and vitamin B-6 (Δ0.2 ± 0.0 mg, P<0.001), but not of niacin (Δ0.5 ± 0.5 mg, P=0.309).

Combined normalised intake of vitamin B-6, B-12 and folate was related to lower homocysteine concentrations in the fully adjusted model (P=0.003). A higher intake of each of the vitamins was related to lower homocysteine concentrations (β vitamin B-6: -0.46 ± 0.26 μmol/L, P=0.084, β folate: -0.003 ± 0.001 μmol/L, P=0.056, and β vitamin B-12: -0.08 ± 0.03 μmol/L, P=0.024). However, homocysteine concentrations were not related to handgrip strength, walk time, or repeated chair rise test time (P>0.10 for all outcomes).

Intake of niacin equivalents was associated with total urinary niacin metabolites (β: 0.53 ± 0.26 μmol/L, P=0.045). Intake of niacin by itself was not associated with the niacin metabolites (β: 0.48 ± 0.33 μmol/L, P=0.147). The sum of the urinary metabolites was not associated with handgrip strength, walking time or chair rise test (P>0.30 for all outcomes).

Excluding energy misreporters (n=28 underreporters, n=0 overreporters) resulted in an attenuated, but still significant, association between vitamin B-6 intake and handgrip strength (with effect modification by physical activity level), and attenuation to insignificance for the association between vitamin B-6 intake and chair rise test time (β -0.031 ± 0.016 s (log), p=0.053, data not shown). Excluding 240 participants who used B-vitamin containing supplements resulted in a slight attenuation of the interaction effects between intake of niacin and physical function in relation to handgrip strength, and an attenuation towards insignificance for the association between vitamin B-6 intake and chair rise test (β -0.024 ± 0.018 s (log), p=0.182, data not shown).

Table 2. Baseline characteristics per tertile of B-vitamin intake. Data presented as mean (SD) unless stated otherwise.

	Niacin			Vitamin B-6			Folate			Vitamin B-12		
	Tertile 1	Tertile 2	Tertile 3	Tertile 1	Tertile 2	Tertile 3	Tertile 1	Tertile 2	Tertile 3	Tertile 1	Tertile 2	Tertile 3
n	415	417	417	416	417	416	416	417	416	349	350	349
Gender (% female)	57	60	52	55	57	56	47	62	59	54	61	57
Age (y)	71.1 (4.1)	70.9 (4.1)	70.7 (3.8)	70.9 (3.9)	71.0 (4.1)	70.8 (4.0)	71.0 (4.0)	71.0 (3.9)	70.7 (4.0)	71.2 (4.1)	71.1 (4.0)	70.8 (3.9)
Height (cm)	166 (9)	165 (9)	166 (9)	166 (9)	165 (10)	165.6 (9)	167 (9)	165 (9)	165 (9)	165 (9)	165 (9)	166 (9)
Weight (kg)	71.9 (12.5)	73.4 (13.8)	75.4 (13.7)	72.8 (12.8)	73.9 (13.8)	74.0 (13.6)	75.8 (12.9)	72.8 (13.8)	72.1 (13.3)	73.4 (12.8)	73.0 (13.3)	76.2 (14.1)
BMI (kg/m^2)	26.1 (3.5)	26.8 (4.1)	27.4 (4.1)	26.3 (3.6)	27.0 (4.0)	26.9 (4.2)	27.0 (3.6)	26.8 (4.2)	26.4 (3.9)	26.9 (3.8)	26.6 (3.9)	27.5 (4.2)
Smoker (%never/former/current)	60/37/3	53/44/3	47/46/7	55/40/2	52/44/4	53/43/4	47/46/7	59/38/3	54/42/4	50/43/7	49/46/4	53/44/3
Drinker (%non/ light/ heavy)[b]	19/63/18	18/68/14	15/67/17	17/63/21	16/69/14	19/67/14	16/64/20	17/69/14	20/66/14	29/55/16	14/70/17	14/71/15
Education (low/medium/high)[c]	13/52/35	15/48/37	12/56/32	18/46/36	15/53/32	8/57/35	18/52/30	12/52/36	11/52/37	23/46/31	11/51/37	7/58/34
PASE-score	134 (54)	131 (56)	133 (56)	133 (58)	129 (53)	137 (55)	131 (56)	133 (55)	134 (56)	124 (59)	135 (50)	139 (58)
Homocysteine (µmol/L)	12.9 (4.7)	12.5 (4.2)	12.5 (3.8)	12.8 (4.3)	12.6 (4.6)	12.4 (3.8)	13.1 (4.4)	12.3 (4.3)	12.4 (3.9)	13.9 (4.7)	12.1 (4.0)	12.2 (3.7)
Niacin intake (mg/d)[a]	13.1 (11.1-14.9)	16.7 (14.9-18.6)	22.1 (19.7-25.8)	14.5 (12.1-17.7)	16.5 (14.0-19.6)	20.2 (17.1-24.4)	16.0 (13.1-19.2)	16.1 (13.4-19.2)	19.3 (15.7-23.4)	15.9 (12.9-19.5)	16.4 (13.5-19.9)	18.6 (15.7-22.3)
Vitamin B-6 intake (mg/d)	1.5 (0.4)	1.7 (0.4)	2.2 (0.6)	1.4 (0.3)	1.7 (0.3)	2.3 (0.6)	1.6 (0.5)	1.7 (0.5)	2.1 (0.6)	1.7 (0.5)	1.8 (0.6)	2.0 (0.6)
Folate intake (ug/d)	264 (82)	281 (91)	323 (106)	248 (77)	278 (82)	342 (104)	208 (49)	274 (43)	387 (87)	272 (102)	271 (78)	307 (103)

Table 2 continued

	Niacin			Vitamin B-6			Folate			Vitamin B-12		
	Tertile 1	Tertile 2	Tertile 3	Tertile 1	Tertile 2	Tertile 3	Tertile 1	Tertile 2	Tertile 3	Tertile 1	Tertile 2	Tertile 3
Vitamin B-12 intake (ug/d)[a]	3.8 (2.6-5.2)	4.2 (2.9-5.9)	4.9 (3.5-8.0)	3.8 (2.5-5.2)	4.2 (3.0-6.0)	4.9 (3.5-7.6)	4.0 (2.6-5.7)	4.3 (3.1-6.1)	4.7 (3.1-7.5)	2.5 (1.9-3.2)	4.2 (3.6-4.8)	7.5 (6.1-10.0)
Protein intake (g/d)	71 (16)	74 (15)	81 (20)	71 (16)	74 (16)	81 (20)	73 (17)	74 (16)	79 (19)	69 (17)	73 (15)	81 (19)
Energy intake (kcal/d)	1901 (457)	1815 (414)	1876 (458)	1912 (461)	1815 (417)	1865 (450)	1882 (463)	1833 (419)	1877 (450)	1849 (448)	1777 (398)	1873 (437)
B-supplement users (%)	21	20	18	18	18	22	14	22	21	18	24	20
Dominant hand grip strength (kg)	30.8 (9.3)	31.2 (9.4)	32.5 (9.6)	30.8 (9.5)	31.4 (9.3)	32.2 (9.6)	32.6 (9.7)	30.8 (9.0)	31.1 (9.6)	31.0 (9.8)	30.7 (9.1)	33.1 (9.7)
Total SPPB score[a]	12 (11-12)	12 (11-12)	12 (11-12)	12 (11-12)	12 (11-12)	12 (11-12)	12 (11-12)	12 (11-12)	12 (11-12)	12 (11-12)	12 (11-12)	12 (11-12)
Repeated chair rise time (s)	10.3 (2.9)	10.3 (2.5)	10.2 (2.8)	10.2 (2.5)	10.5 (3.1)	10.2 (2.7)	10.3 (2.6)	10.4 (3.0)	10.2 (2.7)	10.2 (2.9)	10.4 (2.7)	10.2 (2.7)
Time to walk 2.44 meters (s)	2.5 (0.6)	2.5 (0.6)	2.4 (0.5)	2.5 (0.5)	2.5 (0.6)	2.4 (0.5)	2.4 (0.5)	2.5 (0.6)	2.4 (0.4)	2.4 (0.6)	2.4 (0.6)	2.4 (0.6)

[a]median (IQR) [b] light-drinker, 1 to 14 units per week; heavy-drinker, 14 or more drinks per week. [c] low, 0-8 years; medium, 9-13 years, high, 14 or more years.
BMI, body mass index; PASE-score, score on the Physical Activity Scale for the Elderly; SD, standard deviation; SPPB, short physical performance battery.

Table 3. Association between intake of the four different vitamins and measures of physical functioning.

	Handgrip strength				Walk time (log)				Repeated chair rise time (log)	
	β exposure	P-value	β interaction exposure * PASE-score	P-value	β exposure	P-value	β interaction exposure * PASE-score	P-value	β of exposure	P-value
Niacin										
Model 1	0.07	0.004			-0.002	0.053			-0.001	0.578
Model 2	0.17	0.009	-0.001	0.057	-0.002	0.062			-0.0004	0.743
Model 3	0.15	0.014	-0.001	0.008	0.0001	0.964			-0.001	0.580
Vitamin B-6										
Model 1	1.42	<0.0001			-0.039	<0.001			-0.024	0.058
Model 2	2.71	<0.001	-0.012	0.015	0.001	0.980	-0.0003	0.100	-0.016	0.261
Model 3	1.76	0.018	-0.014	0.003	0.010	0.715	-0.0003	0.141	-0.033	0.043
Folate										
Model 1	0.003	0.083			-0.0001	0.024			-0.0001	0.130
Model 2	0.001	0.702			0.0001	0.560	-0.0001	0.131	-0.0001	0.613
Model 3	-0.002	0.235			0.0001	0.568	-0.0001	0.137	-0.0001	0.292
Vitamin B-12										
Model 1	0.159	0.001			-0.002	0.213			-0.002	0.340
Model 2	0.114	0.020			-0.001	0.442			-0.0004	0.844
Model 3	-0.004	0.936			-0.0014	0.459			-0.003	0.247

Model 1. Adjusted for age and sex.

Model 2. Additional adjustment for energy intake, drinking status, smoking status, education level and physical activity level.

Model 3. Additional adjustment for study centre and protein intake.

PASE-score, Score on the Physical Activity Scale for the Elderly.

Table 4. Association between intake of niacin or B-6 and handgrip strength over the four quartiles of physical activity.

	Quartile 1 (n=311) median pasescore: 72, range: 5-94			Quartile 2 (n=311) median pasescore: 112, range: 94-128)			Quartile 3 (n=317) median pasescore: 146, range: 128-167			Quartile 4 (n=306) median pasescore: 195, range: 167-548		
	Intercept	β exposure	P-value	Intercept	β exposure	P-value	Intercept	β exposure	P-value	Intercept	β exposure	P-value
Niacin – handgrip strength	56.0	0.07	0.181	58.9	-0.02	0.638	61.5	0.02	0.657	57.6	-0.05	0.481
Vitamin B-6 – handgrip strength	56.4	1.5	0.051	59.4	0.2	0.787	61.3	-0.2	0.783	56.8	-1.4	0.041

Presented data is from the model adjusted for age, sex, energy intake, drinking status, smoking status, education level and physical activity level, study centre and protein intake.
PASE-score, Score on the Physical Activity Scale for the Elderly.

Discussion

This study aimed to assess whether dietary intake of niacin, vitamin B-6, B-12 and folate is associated with physical performance in healthy European older adults. We found that higher intake levels of vitamin B-6 were associated with a better chair rise test performance and that vitamin B-6 intake was associated with improved handgrip strength in participants with low physical activity levels. We found no evidence for an association between intake levels of niacin, vitamin B-12 or folate and physical performance, and no evidence for mediation by niacin status or homocysteine concentrations.

Intake of vitamin B-6 was related to higher handgrip strength in participants in the lowest quartile of physical activity. The median PASE-score of these participants was 72, which is considerably lower than recommended levels. A PASE-score of 130 is recommended, as this predicts favourable body composition measures like waist circumference [39]. To increase PASE-score from 72 to 130, one would have to increase activity by as much as two additional hours of moderate to strenuous physical activities on at least five days per week [39]. High levels of physical activity might increase the requirements of vitamin B-6. Vitamin B-6 is needed for glycogen breakdown and amino acid metabolism, two metabolic pathways that are upregulated by exercise [40]. Exercise can lead to lower vitamin B-6 status, which is illustrated by higher losses of vitamin B-6 and increased risks of vitamin B-6 deficiencies in athletes [41]. We have no data on vitamin B-6 status in our study population, but if the relationship between vitamin B-6 intake and status is indeed attenuated in the physically active participants, this could explain the observed interaction effect.

In contrast, the relation between vitamin B-6 and chair rise test performance did not vary by physical activity levels. This discrepancy is interesting, as handgrip strength and chair rise test both reflect limb muscle strength [42], and upper- and lower limb strength are strongly correlated [43]. However, as chair rise test is timed, speed is a factor in this test, while speed does not play a role in handgrip strength. Consequently, the chair rise test reflects muscle power, whereas handgrip strength reflects muscle strength, which might underlie the difference. Future studies should explore the relation between B-6 intake, status and physical performance over different levels of physical activity. It should also be noted that the association of vitamin B-6 intake with chair rise test performance lost significance in the sensitivity

analysis that excluded vitamin B-supplement users. This might be due to the loss of power induced by the exclusion of 240 participants, or caused by low robustness of the B-6-chair rise test association.

Similar to our findings, a role for vitamin B-6 intake in maintaining physical performance has been reported three times before [20, 21, 44]. First, Struijk et al. showed that a higher intake of vitamin B-6 was associated with a lower risk of impaired mobility in *n*=1630 Spanish older adults of the Seniors-ENRICA cohort [20]. Participants in the highest tertile of vitamin B-6 intake (mean ± SD intake: 2.6 ± 0.4 mg/d) had a significantly reduced risk of developing impaired mobility compared to participants in the lowest tertile (mean ± SD intake: 1.5 ± 0.2 mg/d). However, the authors found no evidence for an association between vitamin B-6 intake and physical performance. It should be noted that in their study, physical performance was assessed by questionnaires, which may have introduced bias. Similar to our findings, Struijk et al.[20] did not find associations between vitamin B-12 and folate intake with measures of physical performance. A second study, again in participants of the Seniors-ENRICA cohort, found that participants in the lowest tertiles for vitamin B-6 and folate intake had a more than doubled risk of developing frailty, compared to participants in the highest tertiles [21]. Low intakes of vitamin B-12 did not increase frailty risk in that study. Finally, by applying mixed graphical models to merged data from different cohorts of Dutch older adults (*n*=662), Behrouzi et al. identified direct associations between vitamin B-6 intake and total SPPB score and chair rise test, but not handgrip strength [44]. They also found that intake levels of vitamin B-12 were associated with improved gait speed, and intake levels of folate with total SPPB score.

We hypothesized that vitamin B-induced homocysteine suppression would be the most important mechanism leading to higher levels of physical performance at old age. We did show that the combined intake of vitamin B-6, B-12 and folate was related to lower concentrations of homocysteine. However, we did not observe any relation between homocysteine concentrations and physical performance. This observation is in contrast with previous studies in older adults, which showed that high homocysteine concentrations negatively correlate with strength [9], chair rise test performance [10], and gait speed [11] while adjusting for similar covariates as in our models. Similar to our study, these studies included free-living older adults of >60 [9] >55 [10] or >50 [11] y old. Homocysteine concentrations were either lower than [9], higher than [10], or comparable to [11] homocysteine concentrations in our

study. Physical performance was similar in two of the three studies (walk speed 1.0 ± 0.2 [9] and 1.1 ± 0.2 m/s [11], compared to 1.0 ± 0.2 m/s in our study) and lower in the third study (mean SPPB score 7.4 ± 3.2 [10], compared to 11.3 ± 1.1 in our study). There are no clear indications of why there is no relation between homocysteine concentrations and physical performance in our dataset. However, a prolonged homocysteine reduction could still support the maintenance of a high level of physical performance in our population. This could be tested in a future longitudinal analysis with sufficient cases of reduced physical performance.

Besides lowering homocysteine concentrations, vitamin B-6 plays a role in the metabolism of many amino acids, neurotransmitters and fatty acids [45]. Vitamin B-6 status is related to plasma concentrations of polyunsaturated fatty acids, and short term dietary vitamin B-6 restriction can already decrease plasma concentrations of especially n-3 fatty acids [46]. Moreover, dietary B-6 restriction leads to lower plasma creatine concentrations [47]. Both n-3 fatty acids and creatine concentrations are related to measures of physical performance [48, 49]. Therefore, the role of vitamin B-6 in the metabolism of fatty acids and amino acids that are important for an optimal physical function could be underlying for the associations found in the present study.

The main food sources of vitamin B-6 intake in European adults are meat, dairy products, vegetables, fruits and potatoes. The high contribution of animal foods to vitamin B-6 intake should be noted with regard to our observed associations, as animal foods also contribute to increased intake of high-quality protein. High-quality protein intake is known to improve muscle protein synthesis and might contribute to physical performance [50]. Participants in the highest tertile of vitamin B-6 intake had a mean protein intake of 80.6 g/d, compared to 71.3 g/d in participants in the lowest tertile. We dealt with this issue by adjusting our final models for total protein intake, so that observed associations are independent of protein intake.

Niacin intake was associated with handgrip strength, but differed across levels of physical activity. However, when stratifying the sample by quartiles of physical activity, no significant associations were found. Possibly, stratified associations did not reach significance due to insufficient power, since there were no more than 317 participants included in each quartile. We did observe a significant positive association between dietary intake of niacin equivalents and 24-h urinary excretion of NMN and 2-Pyr, which is in line with previous findings [34]. Niacin intake by itself was not related to the urinary niacin metabolites. This observation illustrates that

tryptophan intake is quantitatively more important for NAD+ availability than niacin intake, which is in line with the literature [51]. Although our data suggest that niacin intake plays a role in physical performance, we are not able to conclude it from our findings. To our knowledge, the association between niacin status and physical performance has not been investigated before. More studies investigating this association are needed, and these studies should test for effect modification by physical activity.

Excluding possible energy underreports resulted in attenuation of the association between vitamin B-6 intake and physical performance. The 28 participants that were excluded did have energy intakes above 500 kcal per day, but below 80% of their estimated basal metabolic rate. As the results of the attenuation imply that these participants also had lower physical performance, it is possible that these participants were not true underreports, but that they were at risk of developing malnutrition. It is, therefore, not warranted to exclude them from the analysis.

This study has some limitations. First, due to the cross-sectional nature, we were not able to draw any conclusions regarding causality. It would be interesting to investigate these associates in a long-term longitudinal study to observe whether intake of B-vitamins plays a role in the prevention of the age-related decline in physical performance. Secondly, physical performance was very good in this cohort. We hypothesize that the associations that we found in this high-functional cohort with little interindividual variance might be larger when studied in a more fragile population. Our sample size was large, and we had excellent exposure and outcome assessment, and outcome measurements were well-standardised over the centres. Finally, a strength is that in our models we adjusted for many important confounders, including protein intake.

To conclude, in this large population of healthy European older adults with a high level of physical performance, we found evidence for positive associations between vitamin B-6 and chair rise test in the full population, and between vitamin B-6 intake and handgrip strength in participants with low physical activity. Homocysteine concentrations did not mediate these associations. We found no evidence for associations between intake levels of niacin, vitamin B-12 and folic acid with physical performance. Vitamin B-6 might be of added value in preventing age-related decline in physical performance, especially in cases where increasing physical activity is not feasible. Further research should focus on the causality and the mechanisms underlying the association between vitamin B-6 intake and physical performance.

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Chapter 6

Dietary intakes of vegetable protein, folate, and vitamins B-6 and B-12 are partially correlated with improved physical functioning of Dutch older adults using Copula Graphical Models

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Abstract

Background In nutritional epidemiology, dealing with confounding and complex inter-nutrient relations are major challenges. An often-used approach is dietary pattern analyses, such as principal component analysis, to deal with inter-nutrient correlations, and to more resemble the true way nutrients are consumed. However, despite these improvements, these approaches still require subjective decisions in the preselection of food groups. Moreover, they do not make efficient use of multivariate dietary data, as they detect only marginal associations. We propose the use of copula graphical models to model and make statistical inferences regarding complex associations among variables in multivariate data, where associations between all variables can be learned simultaneously.

Objective The aim of this study is to reconstruct nutritional intake and physical functioning networks in Dutch older adults by applying a copula graphical model.

Methods We address this issue by uncovering the pairwise associations between variables while correcting for the effect of remaining variables. More specifically, we used a penalized copula graphical model to infer the precision matrix, which contains all the conditional independence relationships between nodes in the graph. The non-zero elements of the precision matrix indicate the presence of a direct association. We applied this method to reconstruct nutrient – physical functioning networks from the combined data of four studies (Nu-Age, ProMuscle, ProMO, V-Fit, *total n* = 662, mean age= 75±7 years).

Results Intakes of vegetable protein and vitamin B-6 were partially correlated with higher scores on total Short Physical Performance Battery (SPPB) and the chair rise test. Intakes of B-12 and folate were partially correlated with higher scores on the chair rise test and total SPPB, respectively.

Conclusions We determined that vegetable protein, vitamin B-6, folate and B-12 intakes are partially correlated with improved functional outcome measurements in Dutch older adults.

Introduction

In studying relations between nutritional intake and health-related outcomes, dealing with confounding is a major challenge. A straightforward and frequently used approach is via linear models, which adjusts for important covariates that confound the association. These covariates, however, have to be selected a priori, and this selection is therefore prone to subjectivity. Many other approaches have arisen to (partly) overcome the issues in dealing with confounders. In the last years, more and more researchers used dietary pattern analyses, such as principal component analysis and cluster analysis, to deal with inter-nutrient correlations, and to more resemble the true way nutrients are consumed. However, despite these improvements, these approaches still require subjective decisions in the preselection of food groups [1]. Furthermore, these approaches only inform about relationships between dietary patterns and outcomes, which do not allow for mechanistic explanations and for identification of the exact nutritional factors that need improvements [2].

Graphical models are powerful class of statistical models for reconstructing the complex relationships between variables in multivariate data [3]. The key feature of graphical models is to uncover *conditional dependence* relationships, meaning that two variables are connected by an edge if and only if they are dependent after all other variables are accounted for. The detection of conditional (in)dependence relationships through graphical models is a key component of the statistical analysis of observational studies. Graphical models, and specifically undirected graphical models, have been used extensively in genetics [4-9], metabolomics [10, 11] and epidemiology [12, 13]. There are different types of graphical models (see for example [14]), one specific type of graphical model is called copula graphical model (CGM), which can deal with ordinal data, non-Gaussian data, and mixed ordinal-continuous data. Gaussian CGM is the simplest possible multivariate ordinal (or mixed ordinal-continuous) model as it uses the least number ($O(p^2)$ for p number of variables or to be precise $p(p-1)/2$) of parameters to describe the full multivariate dependence. Other multivariate models for ordinal data (or mixed ordinal-continuous data) usually require estimating a larger number of parameters, which make them more complex to be applied to high-dimensional data.

Recently, Iqbal et. al. [15] used Gaussian graphical models for identifying conditional independence structures between food intake variables in a dietary intake data to understand the eating behaviour of German adults. The Gaussian graphical model assumes that data follow a multivariate Gaussian distribution. However, multivariate datasets arising from food and nutrition science typically accommodate different variable types. Thus, in this paper, we focus on copula graphical models [16, 17] which can be applied to any study that involves a mixture of binary, ordinal, and non-Gaussian variables. Recently CGMs have been used to detect dietary meal networks (66). Here, we use the methodology developed by Behrouzi and Wit (2019) (18) to learn complex associations patterns that exist among nutrient intake, physical performance, and muscle strength. This method may help to identify conditional intakes of different nutrients to prevent the progressive loss of muscle mass and muscle strength and ultimately to understand the process involved in ageing.

Dietary adjustments might provide feasible strategies to promote healthy ageing. Therefore, understanding the relationships between dietary intake and physical functioning in older adults is of large interest. However, decisions regarding new dietary strategies for healthy ageing cannot be based on the result of a single study, because results typically vary from one study to another. Rather, it is needed to synthesise data across studies. Here we integrated data from multiple studies, where we have combined the most overlapping dietary studies that are included dietary intake, physical performance, and muscle strength, carried out within the same centre (Wageningen University, NL). Given the above consideration, we combined the baseline data of four studies: Nu-Age [18], Promuscle [19], ProMO [20], and VFit [21].

The key objective of this paper is, therefore, to implement the copula graphical model to the combined data (as well as to each individual study) to reveal conditional dependence relationships between muscle function variables (handgrip, different functional tests), nutrients, and other covariates (e.g. age, gender, BMI). Studying underlying partial correlation networks jointly for dietary intake and nutrient components along with muscle strength and physical performance variables may indicate which nutrients play important roles in age-related functional decline.

Methods

Assessment of dietary intake and physical functioning in the four sub-studies

The inclusion and exclusion criteria and the used dietary assessment methods of the four sub-studies are shown in **Table 1**. All subjects in all studies gave written informed consent, and all studies were approved by the Wageningen University Medical Ethical Committee. The sub-studies that started after 2005 Nu-Age, ProMuscle and ProMO) were registered at Clinicaltrials.gov.

Table 1 Characteristics of the four included studies

Study name	**Nu Age**	**ProMuscle**	**ProMO**	**V-FIT**
Years of enrolment	2012-2014	2009-2010	2016-2017	1997
Inclusion criteria	Aged 65-79 y old	> 65 y old, Frail or pre-frail according to Fried criteria	> 65 y old, Malnutrition (score <12 on MNA-SF)	> 70 y old, Using care service, BMI ≤25 kg/m^2 or unintentional weight loss
Exclusion criteria	• Frail according to Fried criteria • Malnutrition • Dementia • Major chronic diseases • Severe heart disease • Insulin-treated diabetes	• Participation in resistance-type exercise programs in 2 y prior to study • eGFR < 60 ml · min^{-1} · $(1.73m^2)^{-1}$ • Any present form of cancer • COPD • Diabetes	• Resistance exercise >2h / week • Life expectancy <12 months • eGFR < 30 ml · min^{-1} · $(1.73m^2)^{-1}$ • Use of diabetes medication • Use of >21 alcohol units per week	• Regular exercise • Institutionalised • Terminal disease • Taking multivitamins
Dietary assessment method	7-day food records	3-day food records	2-day food records	3-day food records

COPD, chronic obstructive pulmonary disease; eGFR, estimated glomerular filtration rate; MNA-SF, mini nutritional assessment tool short form.

Nu-Age

The participants of the Nu-Age trial (*n*=252 Clinicaltrials.gov identifier: NCT01754012) were recruited via invitation letters sent to all older adults living in apartment buildings in the surroundings of Wageningen and Arnhem (The Netherlands). Participants were independently living non-frail older adults aged 65-79 y, free of malnutrition, dementia, major chronic diseases, severe heart disease and type 1 or insulin-treated type 2 diabetes.
Participants filled out food records on seven consecutive days. Participants were trained in reporting foods, portion sizes and preparation methods. Trained dietitians or research nutritionists assessed the food records for completeness during a home visit. Nutrient content was calculated by the Dutch food composition database of 2011. Frequency, type, brand name and dose of specific vitamin supplements (multivitamin, iron, vitamin D, vitamin B complex and folic acid) were assessed via additional questionnaires. SPPB was performed at Wageningen University, in accordance with the protocol described by Guralnik et al. [22], with a 2.44 m gait speed, 5-times chair rise test and a 3-position balance test.

ProMuscle

A total of *n*=122 participants were included in the ProMuscle trial (Clinicaltrials.gov identifier: NCT01109628). Participants were recruited via a volunteer database, flyers and information meetings. Participants were included when frail or pre-frail according to the Fried criteria [23]. Participants were excluded when they participated in resistance-type exercise programs in the 2 y before screening, or when they were diagnosed with any form of cancer, chronic obstructive pulmonary disease, diabetes, or renal insufficiency.
Dietary intake was assessed via 3-day food records on randomly assigned week and weekend days. Food records were discussed with trained dietitians, and household measures were used to optimise the portion size estimation. Nutrient intake was calculated using the Dutch food composition database of 2006. Dominant handgrip strength was measured by hand-held dynamometry (Jamar, Jackson, MI) to the nearest 0.5 kg. Participants were seated on a chair without armrests, with their arms flexed in a 90-degree angle. The maximum effort of three attempts was used for analysis. The SPPB was performed similar to the NuAge study, following the original protocol as described by Guralnik et al. [22].

ProMO

The study population of ProMO (*n*=81, Clinicaltrials.gov identifier: NCT02683720) consisted of participants aged 65 y and older who were malnourished or at risk of malnutrition (a score below 12 on the Mini Nutritional Assessment Tool – Short form). Participants were recruited via dieticians, geriatric outpatient clinics of two hospitals (Rijnstate, Arnhem, The Netherlands and Gelderse Vallei, Ede, The Netherlands), the volunteer database of the university, and via advertisements in local and online media. Exclusion criteria were an expected life-expectancy of <12 months, performing over 2 h / w of resistance exercise, planned increase in exercise during the study, impaired kidney function (estimated glomerular filtration rate <30 ml · min^{-1} · $(1.73m^2)^{-1}$, measured at baseline), lactose intolerance or milk protein allergy, use of corticosteroids (unless administered via inhaler or topically), use of diabetes medications, consumption of >19 portions of oral nutritional supplements in the previous month or >9 in the previous week and use of >21 alcohol units per week. Dietary intake was assessed by using 2-day food records on two consecutive days. Participants were interviewed by trained dietitians to optimise the completeness of the food records. Portion sizes were estimated by using household measures. The Dutch food consumption database 2011 was used to calculate the average daily nutrient intake. Physical functioning was assessed by means of isokinetic hand grip strength and SPPB. Participants performed the handgrip strength test three times per hand, while seated on a chair without armrests and with their elbows flexed in a 90-degree angle. SPPB was assessed via the updated protocol of Guralnik et al. [24], with a balance test, 5-times chair rise test and a 4 m gait speed test.

V-Fit

Participants of the V-Fit trial (*n*=207) were recruited via personal letters sent to senior residences, home care organisations, general practitioners and local advertisements. Eligible participants were aged 70 y and older, used care services, did not regularly exercise, had a BMI of ≤25 kg/m^2 or experienced unintentional weight loss. Excluded were participants who were institutionalised, had a terminal disease, or were taking multivitamin supplements in the preceding month.
Dietary intake was assessed by a 3-day food record (two weekdays and one weekend day). Participants were visited at home before and after the dietary intake assessment, to explain the procedure and to check completeness. The volumes of frequently used household measures were assessed to optimise portion size

estimation. Nutrients were calculated by using the Dutch food consumption table of 1997. Physical functioning was measured by means of peak dominant hand grip strength and the Groningen Fitness test for Elderly. The latter is comparable to the SPPB, as it contains a balance test, five-times chair rise test and a 6-meter gait speed test.

Statistical methods

Graphical Models

Graphical models are a marriage between probability theory and graph theory. They provide a powerful tool for dealing with uncertainty and complexity in statistics. Let $Z = (Z_1, Z_2, \ldots, Z_p)$ denote a random vector with a joint distribution $p(Z)$. The conditional independence relationships among random variables can be summarised in a graph $G = (V, E)$, where V is a set of vertices (or nodes), and each vertex corresponds to a random variable, and E is a set of undirected edges. If two vertices $Z_i, Z_j \in V$ form an undirected edge then we say that Z_i and Z_j are adjacent or connected and write $(i, j) \in E$. Here undirected means that $(i, j) \in E$ is equivalent with $(j, i) \in E$. Thus, undirected graphs represent symmetric relationships. The absence of an edge between Z_i and Z_j corresponds with the conditional independence of the two random variables given the remaining variables under $p(Z)$ and is defined by

$$Z_i \perp\!\!\!\perp Z_j \mid X_{V\setminus\{i,j\}}.$$

This is called the pairwise Markov property**.** Let $X_{n\times p}$ be the data matrix with p the number of variables in the network, and n be the number of observations for each variable. In Gaussian graphical models, it is assumed that the vector of $X = (X_1, X_2, \ldots, X_p)$ follows a p-variate normal distribution $N_p(\mu, \boldsymbol{\Omega}^{-1})$ with mean μ and variance-covariance matrix $\Sigma = \boldsymbol{\Omega}^{-1}$, where $\boldsymbol{\Omega}$ represents the precision matrix. The partial correlation coefficients $\rho_{ij|rest}$, which measure the correlations between X_i and X_j conditional on all other variables in the models, can be calculated as

$$\rho_{ij|rest} = -\frac{\omega_{ij}}{\sqrt{\omega_{ii}}\sqrt{\omega_{jj}}} \qquad (1)$$

where ω_{ij}, $i, j = 1, \ldots, p$ are the elements of the precision matrix $\boldsymbol{\Omega}$.

Copula Graphical Models

Copula graphical models (CGMs) are a flexible type of graphical model, where we relax the multivariate normal distribution assumption for the joint distribution of $X = (X_1, X_2, \ldots, X_p)$. In fact, it can be applied to different types of datasets, such as non-Gaussian data, ordinal data, count data, and a mixed of ordinal-and-continuous data . Mixed types of variable are common in food and nutrition datasets. In the proposed copula graphical model we assume that there is an ordering for the possible values of each observed variable $X_v, v \in V$. This assumption holds if X_v is binary, categorical with ordered categories, count or continuous. We assume the dependence structures among the observed variables $X = (X_1, X_2, \ldots, X_p)$ are given by the Gaussian copula with $p \times p$ correlation matrix. The Gaussian copula model can be constructed by introducing a vector of latent variables $Z = Z_v \sim N_p(0, \boldsymbol{\Omega})$ that are related to the observed variables $X = X_v$ as

$$X_v = F_v^{-1}(\Phi(Z_v)),$$

where F_v denotes the marginal distribution of X_v which are treated as nuisance parameters, and Φ is the cumulative distribution function of the standard normal distribution. As proposed by Behrouzi and Wit [25], we use copula graphical models to perform inference in the parameter $\boldsymbol{\Omega}$ of the Gaussian copula, which contains all the conditional independence relationships between latent variables Z. The inference procedure of their approach is based on penalised Expectation-Maximization (EM) algorithm, which iteratively computes the conditional expectation of the joint log-likelihood in the E-step, and optimises this conditional expectation in the M-step. Furthermore, an ℓ_1 regularisation technique is used to put a grid of penalty parameters on the off-diagonal elements of $\boldsymbol{\Omega}$, which leads to a sparse matrix. The zeroes of $\boldsymbol{\Omega}$ corresponds to the missing edges in the graph. A grid of regularisation parameters $\Lambda = (\lambda_1, \ldots, \lambda_N)$ determines the level of the sparsity of $\boldsymbol{\Omega}$. Different penalty results in a different graph structure. One approach to select an optimal graph is to compute various information criteria based on the observed penalised maximum log-likelihood. Since we are interested in graph estimation, we use the extended Bayesian information criterion (eBIC) [26] that has been introduced as follow

$$eBIC(\lambda) = -2\, l(\widehat{\boldsymbol{\Omega}}_\lambda) + \{\log(n) + 4\gamma \log(p)\} df_\lambda,$$

for conditional independence graph selection. Here, df_λ refers to the number of non-zero elements in the off-diagonal of $\widehat{\boldsymbol{\Omega}}_\lambda$ and $\gamma \in [0,1]$ determines the strength of

prior information on the size of the model space. We set $\gamma = 1/2$, as it is shown by Foygel and Drton (2010) [26], for moderately large n, the extended BIC with $\gamma = 1/2$ performs well to recover the underlying true graph. The optimal model has the minimum value of eBIC with respect to λ.

Inference Uncertainty

In high-dimensional cases, there is often considerable uncertainty in the number of non-zero elements in the precision matrix. To compute uncertainty associated with the estimation of precision matrix, we used a non-parametric bootstrap method to determine the statistical accuracy and the importance of each link in the estimated nutrients-physical functioning network. One can then choose only those links (or direct associations) that have a high probability of being presence across all the bootstrap samples. For the penalised CGMs, we replicated B datasets that are generated by sampling with replacement from the dataset $X_{n\times p}$. For each replicate, we run the entire inference procedure of the CGMs, including the model selection to estimate an optimal graph. This non-parametric bootstrap reflects the underlying uncertainty in the estimated network. We have implemented this procedure to calculate the uncertainty associated with the estimation of nutrients-physical functioning networks in the combined data.

Analysis

The combined dataset of the four studies contains $n = 662$ individuals and $p = 33$ variables, which we grouped the variables into three categories - physical functioning, nutrient intakes, and general covariates. Different colours in the network represent variables and their corresponding categories (**Figure 1**, results). We note that in the combined dataset we kept the unit of all variables consistent across various studies (e.g. energy intake is in kJ across all the four studies). Furthermore, we defined four dummy variables to represent each study to let the conditional independence property correct for associations that may arise from the different studies.

Copula graphical models were used to construct the underlying connectivity networks for the combined data as well as for the individual studies. For each dataset, we used our R package ***nutriNetwork*** to implement the CGM. More specifically, we used the ***nutriNetwork*** function with the default arguments. Furthermore, to select an optimal network across a grid of penalty parameters, we run the ***selectnet***

function on the output object of *nutriNetwork* function. The sparsity of the selected network is 0.23, where the sparsity level of the highest penalty term proposed by *nutriNetwork* is 0.05. To visualise networks we used ***plot*** function in *nutriNetwork*.

Furthermore, we used a non-parametric bootstrapping approach to determine the uncertainty associated with the estimated links in the nutrients-physical functioning networks from the combined data. We generated 200 independent bootstrap samples from the combined data, as we described above. For each bootstrap sample we run the entire inference procedure, including the model selection, using *nutriNetwork* package. Furthermore, we calculated the frequency of each non-zero element of $\widetilde{\boldsymbol{\Omega}}^{(b)}_{\hat{\lambda}}$ $(b = 1, \dots, 200)$ whose is also non-zero in the estimated $\widehat{\boldsymbol{\Omega}}_{\hat{\lambda}}$ from the original combined data. In addition, we measured the fit of our model to the combined data**.** In **Supplementary Table 1** we show that the proposed copula graphical model fits the data properly.

Results

Baseline characteristics of all participants included in the analyses are presented in **Table 2**. Characteristics of the participants in the four trials were very comparable. Nonetheless, participants in the ProMO trial had a lower BMI compared to participants in the other three studies, and participants in the Nu-Age trial scored higher on the physical functioning tests (handgrip strength, total SPPB score and chair rise test). The total sample consisted of more female than male participants, and participants scored relatively high on the SPPB, with an average score of 9.6 out of 12. Nutrient intake of the participants over the different trials is depicted in **Table 3**. Overall, the average intakes of nutrients were close to their recommended daily allowances (RDAs) for Dutch older adults [27]. Only intake levels of calcium (average intake 992 mg/d, RDA 1200 mg/d [28]), folate (average intake 243 µg/d, RDA 300 µg/d [29]) and vitamin D (average intake 3.6 µg/d, RDA 20 µg/d [30]) were considerably lower than the RDA. However, the intake of nutrients via dietary supplements is not included in these intake levels. The average daily energy intake in the V-Fit study was lower than in the other trials, but the intake levels of the nutrients were comparable across all studies.

Table 2 Baseline characteristics of included Dutch older participants (n=662)

Characteristics[1]	**Combined** *n*=662	**Nu-Age** *n*=252	**ProMuscle** *n*=122	**ProMO** *n*=81	**V-Fit** *n*=207
Age (y)	75 ± 7	71 ± 4	79 ± 8	74 ± 6	78 ± 6
Sex (% female)	61.7%	55.6%	60.7%	50.6%	74.4%
BMI (kg/m^2)	25.3 ± 3.8	26.1 ± 3.6	27.3 ± 4.2	22.0 ± 3.1	24.4 ± 2.7
Height (cm)	167 ± 9	169 ± 8	166 ± 9	169 ± 9	164 ± 9
Weight (kg)	71 ± 13	75 ± 13	75 ± 13	63 ± 11	66 ± 10
Alcohol intake (units/wk)	9.4 ± 12.3	12.8 ± 11.9	10.1 ± 12.1	6.4 ± 5.1	5.4 ± 13.1
Smoker (% yes)	30.9%	49.6%[2]	5.8%	60.5%[2]	10.8%
Handgrip (kg)	26 ± 9	29 ± 9	26 ± 9	21 ± 10	23 ± 8
SPPB total[3]	9.6 ± 2.5	11.3 ± 1.1	8.0 ± 3.0	10.6 ± 2.1	8.1 ± 1.8
SPPB balance[3]	3.5 ± 0.9	3.8 ± 0.6	3.1 ± 2.1	3.7 ± 0.8	3.5 ± 0.9
SPPB chair[3]	2.6 ± 1.4	3.6 ± 0.7	1.9 ± 1.4	3.2 ± 1.1	1.3 ± 0.6
SPPB gait[3]	3.5 ± 0.9	3.9 ± 0.4	3.0 ± 1.1	3.7 ± 0.6	3.2 ± 1.1

BMI, body mass index. SPPB, short physical performance battery. [1] Data presented as mean ± SD, unless stated otherwise. [2] In ProMO and Nu-Age data, percentage smokers includes current and former smokers. [3] SPPB data reflects score in points out of a maximum of 12 (total) or 4 (balance, gait, chair rise test)

Figure 1 represents the estimated network for the combined data. It shows interesting links between certain nutrients and functional outcome measures. Vitamin B-6 (pyridoxine), folate and vegetable protein intake were positively and directly correlated with total SPPB score, with respectively 88%, 53% and 98% certainty based on bootstrap analysis. Vegetable protein and vitamin B-6 were also positively and partially correlated with chair rise test score (resp. 100%, 61% certainty), and this also holds for vitamin B-12 (cobalamin, 50% certainty). These nutrients were not directly correlated with balance and gait scores, the two other parts of total SPPB score. Based on the estimated network there is no direct association between handgrip strength and any of the nutrients. The proposed method shows that higher age and being female resulted in lower handgrip strength. Height, weight and, surprisingly alcohol intake were positively and directly correlated to handgrip strength. Besides having a lower handgrip strength, being female was also partially correlated to lower alcohol intake, lower energy intake and lower starch intake. Intake of folate was partially correlated with a negative effect to BMI, while intake of vitamin B-12 was positively linked to body weight. In **Supplemental Figure 1**, we reported the corresponding relative frequencies of each link across the 200 bootstrap samples. In particular, almost in all bootstrap samples we inferred a direct link between vegetable protein (veg.pro) and SPPB total (an index for measuring physical functioning). In other words, vegetable protein and SPPB total are partially correlated after adjusting for the effect of all remaining variables.

The ProMO study, which included malnourished participants, was negatively correlated to BMI, after controlling for remaining variables. Intake of vegetable protein was positively and partially correlated with intake of magnesium, starch and fibre. Intake of folate was positively and partially correlated with intake of fibre and vitamin C, and intake of calcium was partially correlated to intake of vitamin B2 (riboflavin).

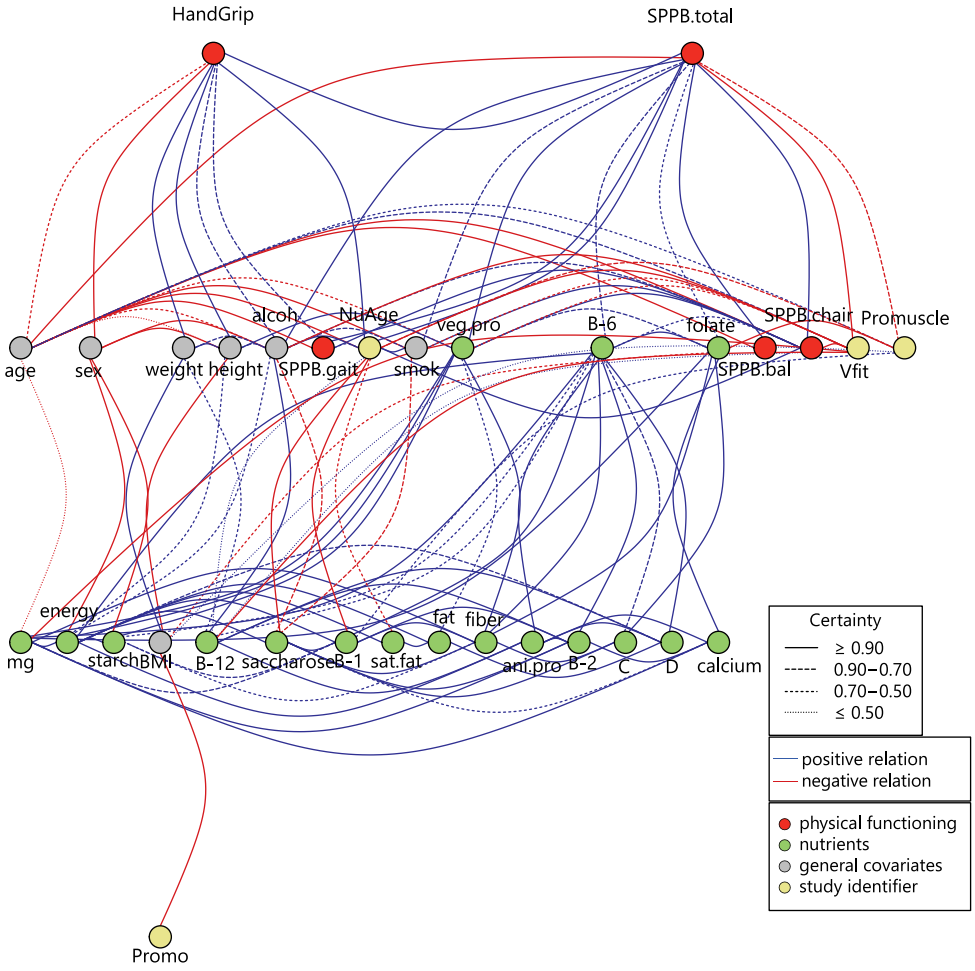

Figure 1 Nutrients-and-muscle-health networks for the combined data. This Figure shows simultaneous associations between nutrient intakes, measures of physical functioning, and the general covariates, which is inferred from copula graphical models. Edges represent conditional dependencies between nodes revealed by partial correlation coefficients. Absence of an edge between two nodes shows conditional independence relationship between them. The type of line used represents the certainty of link, which is the frequency in which the conditional dependence relationship (partial correlation) is found over 200 independent bootstraps. Alcoh, alcohol; ani pro, animal protein; B-1, thiamine; B-6, vitamin B-6; B-12, vitamin B-12; bal, balance; BMI, body mass index; C, vitamin C; D, vitamin D; mg, magnesium; sat. fat, saturated fat; SPPB, short physical performance battery; veg. pro, vegetable protein.

Table 3 Nutrient intake levels of the Dutch older adults (n=662) in the four sub studies

Characteristics[1]	**Combined data**	**Nu-Age**	**ProMuscle**	**ProMO**	**V-Fit**
	n=662	*n*=252	*n*=122	*n*=81	*n*=207
Energy (kcal/d)	1913 ± 469	1908 ± 411	1964 ± 530	2177 ± 507	1779 ± 434
Animal protein (g/d)	46 ± 15	46 ± 13	50 ± 18	50 ± 16	43 ± 14
Vegetable protein (g/d)	28 ± 9	30 ± 8	26 ± 7	33 ± 12	23 ± 7
Fat (g/d)	75 ± 24	73 ± 20	78 ± 25	91 ± 30	70 ± 22
Saturated fat (g/d)	30 ± 11	27 ± 9	33 ± 11	37 ± 14	30 ± 10
Starch (g/d)	105 ± 33	109 ± 31	100 ± 36	121 ± 40	96 ± 29
Sugar (g/d)	106 ± 41	92 ± 32	122 ± 51	106 ± 36	114 ± 41
Fiber (g/d)	22 ± 7	22 ± 6	22 ± 7	24 ± 9	21 ± 7
Thiamine (mg/d)	1.0 ± 0.5	0.9 ± 0.3	1.2 ± 0.7	1.1 ± 0.5	1.1 ± 0.6
Riboflavin (mg/d)	1.4 ± 0.5	1.4 ± 0.4	1.6 ± 0.6	1.6 ± 0.6	1.3 ± 0.5
Vitamin B-6 (mg/d)	1.5 ± 0.7	1.6 ± 0.5	1.6 ± 0.8	1.6 ± 0.8	1.4 ± 0.9
Folate (µg/d)	243 ± 85	255 ± 71	189 ± 68	300 ± 115	239 ± 78
Vitamin B-12 (µg/d)	4.5 ± 2.7	5.0 ± 2.7	4.7 ± 2.9	5.0 ± 3.1	3.5 ± 2.2
Vitamin C (mg/d)	104 ± 54	101 ± 48	113 ± 62	121 ± 68	95 ± 45
Vitamin D (µg/d)	3.6 ± 2.3	3.6 ± 2.2	4.6 ± 3.1	3.6 ± 2.2	3.1 ± 1.7
Calcium (mg/d)	992 ± 346	965 ± 309	1032 ± 394	1124 ± 386	946 ± 327
Magnesium (mg/d)	318 ± 90	333 ± 77	329 ± 83	377 ± 104	267 ± 80

[1] Data are presented as mean ± SD.

Visual overviews of the CGMs for the different sub-studies are presented in the supplementary data (**Supplementary Figures 2-5**), and corresponding partial correlation coefficients are presented in **Supplementary Figures 6-10**. **Table 4** provides an overview of the direct links between nutrients and measures of physical functioning the models revealed in the combined data (letter A) and in the four different sub-studies (letters B – E). Note that in all datasets the model did not reveal links between intake of animal protein, calcium or magnesium and the functional outcome measures. Handgrip strength was partially correlated to intake of energy, vegetable protein, thiamine, total fat and saturated fat in sub-studies, but these links were not identified in the combined data. The other way around, intake of folate and B-12 intake was not linked to functional outcomes in the sub-studies, but were partially correlated to total SPPB score and chair rise test in the combined data.

Nu-Age

The network of the Nu-Age (B, supplementary Figure 2) study did not suggest any direct link between nutrients and functional outcome measures. In the Nu-Age network, the different SPPB components were only linked to each other, but not to any nutrient or general variable. This isolation can be explained by the average near-maximum scores on all SPPB components, in combination with the subsequent lack of variation. The absence of the expected links between SPPB components and handgrip strength underline the profound isolation. Interestingly, age is not linked to handgrip strength. This is possibly caused by the relatively low mean (71 y) and max age (79 y) in the Nu-Age trial.

ProMuscle

The network based on data of the ProMuscle (C, supplementary Figure 3) study revealed interesting links between nutrients and function measures, which are often not observed in the other studies. Total fat and saturated fat intake, as well as intake of thiamine and energy intake, were positively and partially correlated with handgrip strength. The data of ProMuscle revealed a positive partial correlation of fibre intake with chair rise test, gait speed, and total SPPB scores. The network based on ProMuscle data was the only network that revealed a link between a nutrient and balance score. Vegetable protein was partially correlated with a positive effect to this balance score, as well as to total SPPB score after removing the effect of remaining variables. Total energy intake was partially correlated with total SPPB score, and this

was also identified by the estimated physical functioning networks based on the combined data.

ProMO

The network of the ProMO (D, supplementary Figure 4) study showed a positive partial correlation between vegetable protein intake and handgrip strength. Besides, vitamin B-6 intake showed to be positively related to chair rise test score and total SPPB score, which was also identified by the network of the combined studies. The network based on ProMO data was the only network that identified direct links between vitamin D and physical functioning. Vitamin D was positively correlated to gait speed and total SPPB score, after removing the effect of remaining variables.

V-Fit

Also in the network of the V-Fit (E, supplementary Figure 5) study, positive correlations between vegetable protein intake and handgrip strength and vegetable protein intake and total SPPB score were observed. Here, total energy intake was also positively related to handgrip strength. The other nutrients in the V-Fit study were not partially correlated to physical functioning measures.

Table 4 Overview of all direct links between nutrients and measures of physical functioning in Dutch older adults (n=662) in the combined and separate trials

	Energy	Animal protein	Vegetable protein	Total fat	Saturated fat	Fibre	Thiamine	Vitamin B-6	Folate	Vitamin B-12	Vitamin D	Calcium	Magnesium
Handgrip Strength	c,e		d, e	c	c		c						
Total SPPB	c		A, c, e			c		A, d	A		d		
Balance			c										
Gait						c					d		
Chair			A			c		A, d		A			

A, combined data; b, Nu-Age (no direct links); c, ProMuscle; d, ProMO; e, V-fit.

Discussion

The field of nutritional epidemiology faces more and more criticism on the used methodological approaches. In nutritional epidemiology, associations are mainly assessed via conventional methods like univariate regression [31-33] and principal component analysis [34-36]. Associations between nutrient intake and health outcomes are prone to be confounded by other variables, and intake levels of different nutrients are often partially correlated with each other [37]. Copula graphical models, which are frequently used in various research areas like genomics and metabolomics, can deal with these common caveats in the field of nutritional epidemiology.

A main advantage of the copula graphical model is its ability to distinguish between direct and indirect associations among dietary intake variables. This is due to the estimation of precision matrix (inverse of variance-covariance matrix) that contains all conditional (in)dependence relationships among variables. Conventional methods such as PCA analysis use marginal correlations that do not adjust for the indirect effect of other variables when assessing pairwise associations. Removing indirect effects is crucial in association studies, as it advances our understanding of underlying mechanism that generated data. Sparse structure learning is another advantageous of this study which facilitate interpretation of results. The proposed method is an exploratory tool that can be applied to dietary intake non-Gaussian data and mixed ordinal-continuous data to uncover conditional independence networks between dietary variables. Also, the method can deal with missing values, which are common in nutritional epidemiology, due to the employed EM algorithm. In addition, the conventional methods only adjust for preselected confounding variables [38]. Whereas, the proposed copula graphical model avoids the pre-selection step. In fact, it handles high-dimensional data, where the number of variables can exceed the number of samples. As a result, it addresses confounding by using conditional dependence relationships (or *partial correlations*), where measured associations are adjusted for the influence of all remaining variables in the dataset. The major advantage of the proposed CGM over conventional methods is its ability to distinguish between direct associations (conditional dependence relationships), and indirect associations (marginal dependencies or Pearson correlations). Thus, applying the proposed CGM in nutritional research is appealing, as it addresses the main caveats in nutritional epidemiology by detecting direct associations.

In this study, we used the proposed CGM to detect simultaneous associations between nutrients and measures of physical functioning. Via this method, we detected interesting links between nutrient intake and physical functioning. First, the estimated network of the combined studies shows that intake of vegetable protein plays a role in physical functioning, as it was related to improved total SPPB score and chair rise test score. Interestingly, we do not observe any direct links between animal protein intake and physical functioning, while animal protein is often considered a better source to promote physical functioning than vegetable protein.

Longitudinal studies have found that high animal protein intake attenuates strength loss over six years of follow-up [39], and, if combined with physical activity, decreases the risk of developing disabilities [40]. Based on current knowledge, the consensus is that animal protein has a higher potency in stimulating muscle protein synthesis than vegetable protein [41]. This superiority of animal protein over vegetable protein is characterised by a number of studies comparing animal protein sources with soy protein or wheat protein [42-47]. In a recent review by Gorissen et. al., the authors emphasise the large differences in amino acid profiles of various plant sources [48]. They point out that specific plant-based proteins (for instance potato protein) have an amino acid composition that is much more similar to that of animal protein than the amino acid profile of wheat and soy protein. Our data are based on food intake of exclusively Dutch older adults [49]. Food consumption research in this population shows that less than 1% of their vegetable protein intake is soy-based. Although wheat protein is, with around 50%, the largest source of vegetable protein intake in this population, there are other protein sources with a notable contribution to vegetable protein intake (vegetables 9%, potatoes 7%, fruits and nuts 7%). Therefore, it is possible that the suspected superiority of animal protein over vegetable protein does not fully apply to this dataset of an almost non-soy consuming population.

Besides, it is even possible that vegetable protein has beneficial effects over animal protein on muscle health. The alkaline properties of vegetable protein-containing foods are hypothesised to prevent the muscle wasting response to acidosis, thereby conserving muscle mass during ageing [50]. Acute supplementation with the alkaline bicarbonate resulted in a modestly improved lower extremity strength in older women, but not in men [51]. Sahni et. al. showed that animal protein was positively associated with leg lean mass, but vegetable protein was associated with increased quadriceps strength [51]. The authors suggest that vegetable protein might be linked to improved muscle strength (independent of muscle mass), or that vegetable intake

behaves as a marker of overall dietary quality. This latter explanation is strengthened by their finding that adjustment for fruit and vegetable intake attenuates the association between vegetable protein intake and quadriceps strength. In our study, vegetable protein intake was related to chair rise test, a test which relies primarily on quadriceps strength [52]. Although our networks did not include adjustments for fruit and vegetable intake, we did adjust for dietary fibre and a broad range of other nutrients, which removes large amounts of dietary quality confounding. Still, overall dietary quality could confound this association, as the model did not include data on intake of other compounds in the food matrix of vegetable protein containing products.

All in all, the positive and direct association of vegetable protein (and not animal protein) with physical functioning in this study can be explained via: 1) a relatively high ingestion of higher quality vegetable protein in this Dutch population, 2) muscle mass conserving properties of the alkaline properties of vegetable protein-containing foods, 3) other, yet to be unravelled mechanisms via which vegetable protein may improve muscle quality.

Second, and similar to intake of vegetable protein, intake of vitamin B-6 was also directly associated with total SPPB score and chair rise test score. Two other B vitamins, folate and vitamin B-12, were linked to total SPPB score and chair rise test score, respectively. The exact roles of these B-vitamins in physical functioning are unclear. However, we know that vitamin B-6, B-12 and folate act together in the one-carbon pathway, via which they lower homocysteine levels. Homocysteine levels have shown to be negatively associated with quadriceps strength [53], chair rise test [54] and gait speed [53-55]. A few studies suggest that elevated homocysteine levels can hamper physical functioning by inducing mitochondrial dysfunction [56-58]. Besides, elevated homocysteine levels cause endothelial dysfunction via increased oxidative stress [59], and are associated with increased white matter hyperintensities, which especially affects lower extremity functioning [60, 61]. This is in line with our findings, where no association was found with handgrip strength, but only with functional outcomes related to the lower extremities.

Previous studies assessing the relationship between the intake of these B-vitamins with physical functioning show conflicting results. Intake levels of vitamin B-6, but not folate and B-12, were associated with improved mobility [62]. Alternatively, intake of B-6 and folate, but not B-12, were associated with a lower frailty risk in older adults [63], and intake levels of B-6 and folate, but not B-12, were lower in sarcopenic

vs nonsarcopenic older adults [64]. Considering the clear associations of homocysteine levels and physical functioning, and the role of intake of B-6, folate and B-12 in the one-carbon metabolism, it is likely that adequate intake of these vitamins helps in maintaining physical functioning. The different findings over the studies investigating the association between B-vitamin intake and physical functioning could mean that over the different populations different B-vitamins were limiting in the one-carbon metabolism. Otherwise, it could mean that these individual B-vitamins act on additional, yet to be identified mechanisms in relation to physical functioning.

Besides the use of the CGMs, this study has another major strength: the assessment of dietary intake data via multiple-day food records. This is considered an accurate way to collect data on intake of multiple nutrients, performing better than other frequently used methods as 24h recalls or food frequency questionnaires [65]. A drawback of this study is that data on some confounders were not available. In the design phase of the sub studies, decisions were made on which outcomes to measure. These decisions can be viewed as a form of priori confounder identification that is still present within our approach. Most importantly, we did not have data on physical activity, which can be a major confounder in the intake – function relationship. We did however partly overcome this issue by correcting for total energy intake and body mass index– which together roughly resemble physical activity level.

There are some potential limitations of the proposed copula graphical model. First, non-ordinal categorical variables such as occupation types, race, and religions cannot be taken into account explicitly. However, it is possible to include them into the model via dummy variables. Second, estimation of graph structure is a challenging problem in graphical models where a range of tuning parameters control the sparsity level of a graph. Different methods (e.g. extended Bayesian/Aikake information criteria, cross-validation, and stability selection approach, etc.) have been proposed for structure learning in graphical models. However, different methods may differ by having a few additional or fewer links. Thus, it is always sensible to measure underlying uncertainty of estimated parameter. Third, Gaussian copula graphical models often employ one parameter to model the interaction between a pair of variables and no parameters are available for higher order interactions between three or more variables. This is computationally convenient,

clearly, but this may not correspond to reality. In fact, it is always sensible to do post-hoc checks to see if the fitted model provides a satisfactory fit to the data.

In conclusion, Gaussian copula graphical models are a powerful exploratory method that can be used to reconstruct dietary intake networks from high-dimensional data. They reveal partial correlations between dietary variables which may help us to identify important nutritional factors in age-related declines in physical functioning. In this study we found that vegetable protein, vitamin B-6, folate and B-12 intake were partially correlated with improved functional outcome measurements in Dutch older adults.

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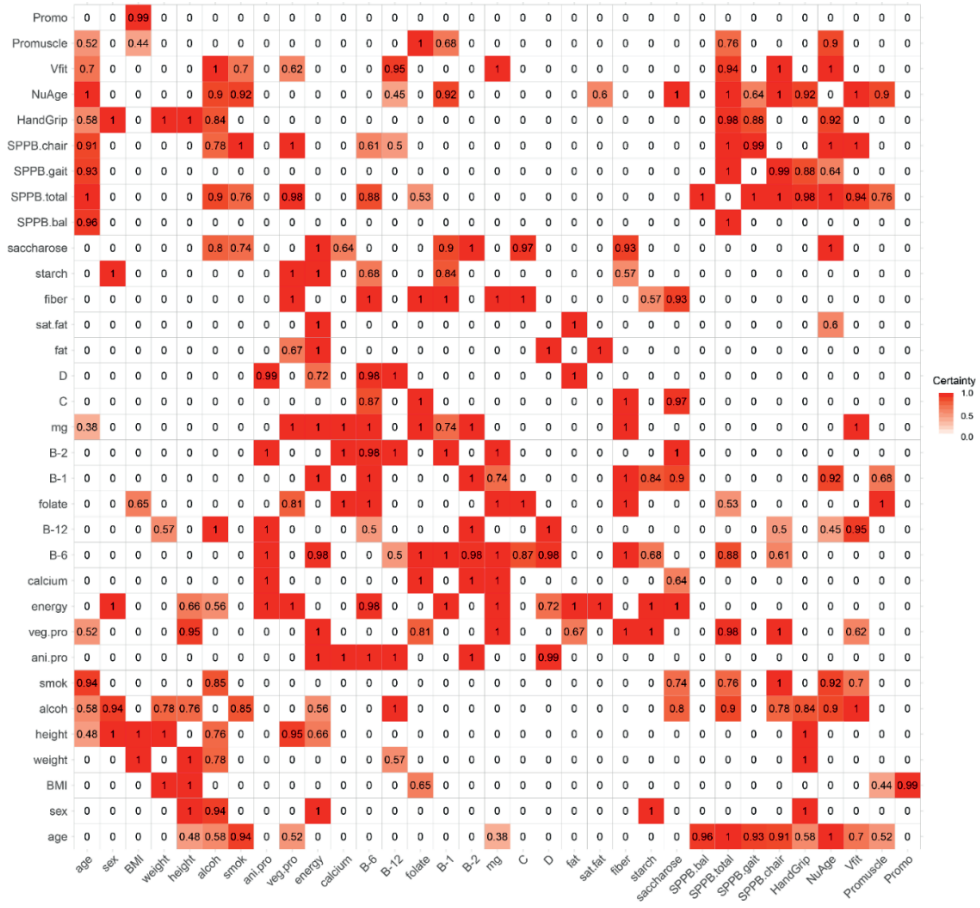
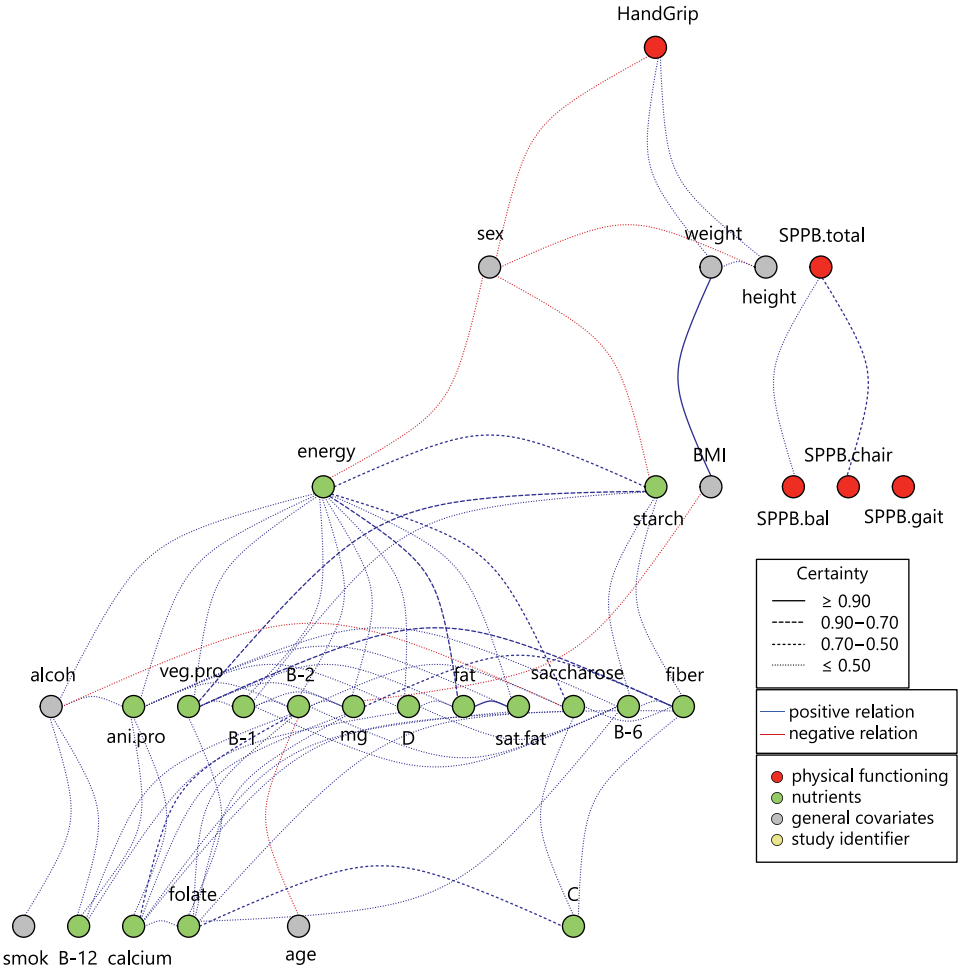
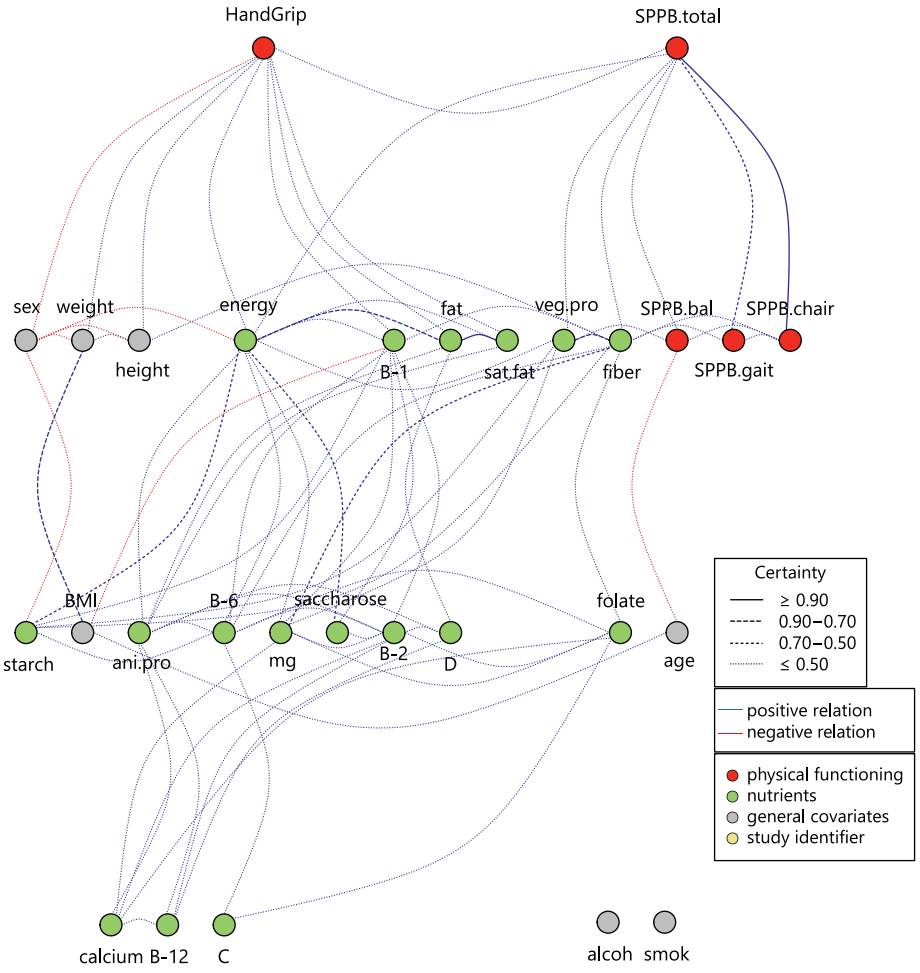
Supplementary Data



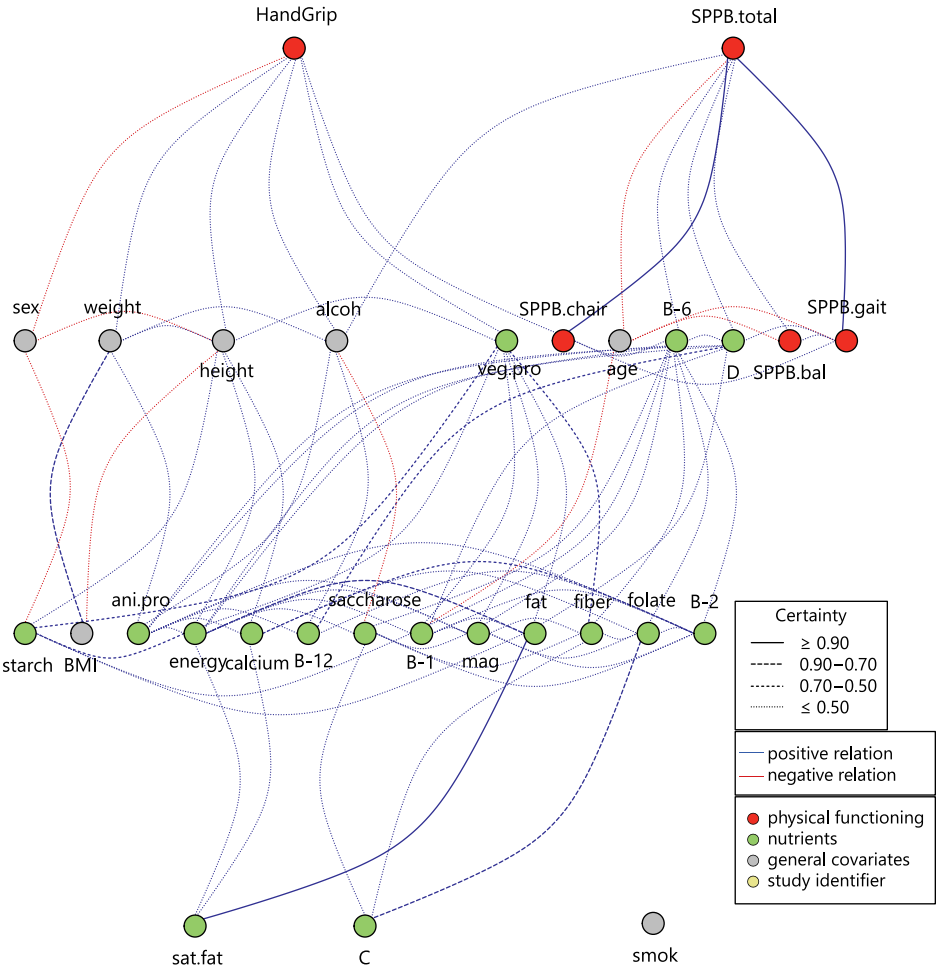
Supplemental Figure 1 The uncertainty associated with each link in the estimated network for the combined data (*n*=662) using a non-parametric bootstrap. Each element represents the relative frequency of having link between variables in bootstrap version of the combined data. Here, we generated 200 independent bootstrap samples. For instance, direct link between vegetable protein (veg.pro) and SPPB total was presented in 98% of all bootstrap samples, and link between vegetable protein and B9 was presented in 81% of all bootstrap samples.



Supplemental Figure 2 Conditional dependencies networks among variables in the NuAge study (*n*=252). The colour of links depicts positive (blue) and negative (red) partial correlations. The type of line used represents the strength of each edge based on partial correlation values. In this graph, physical functioning items (SPPBs) are isolated from the rest of the networks.

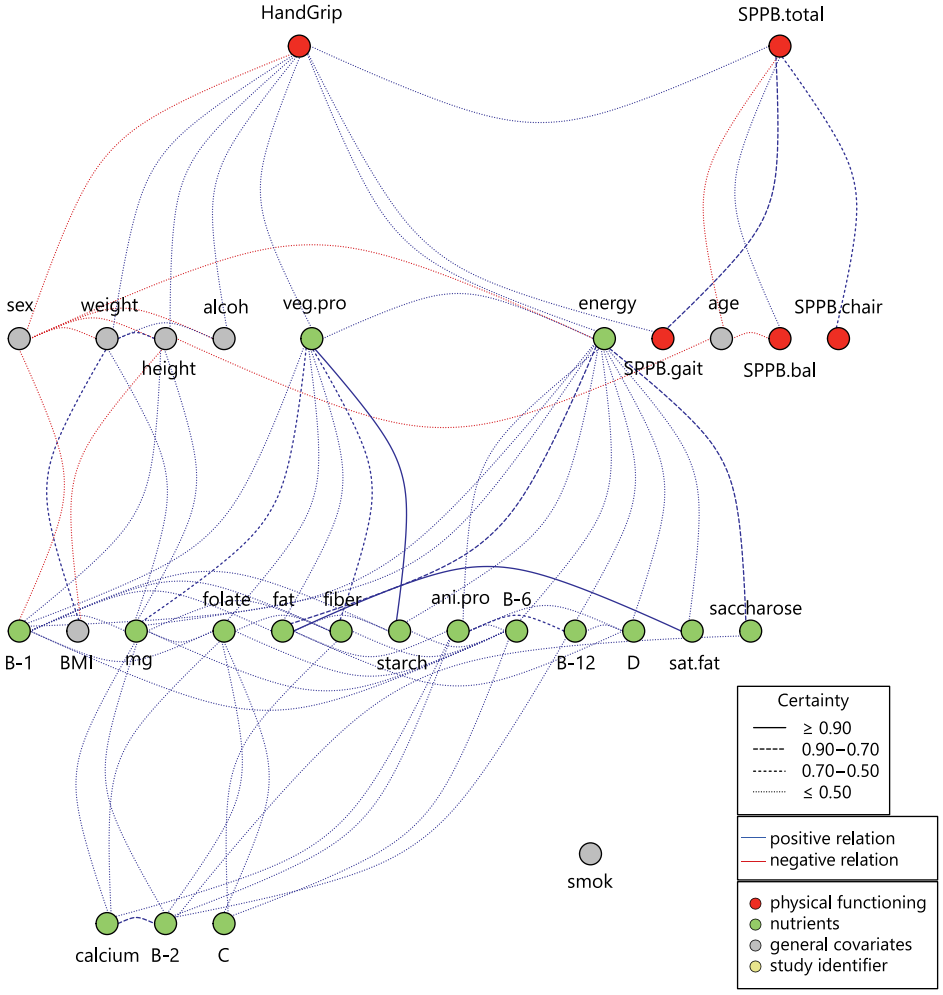


Supplemental Figure 3 Conditional dependencies networks among variables in the ProMuscle study (*n*=122). The colour of links depicts positive (blue) and negative (red) partial correlations. The type of line used represents the strength of each edge based on partial correlation values. In this graph, physical functioning items (SPPBs) are isolated from the rest of the networks.



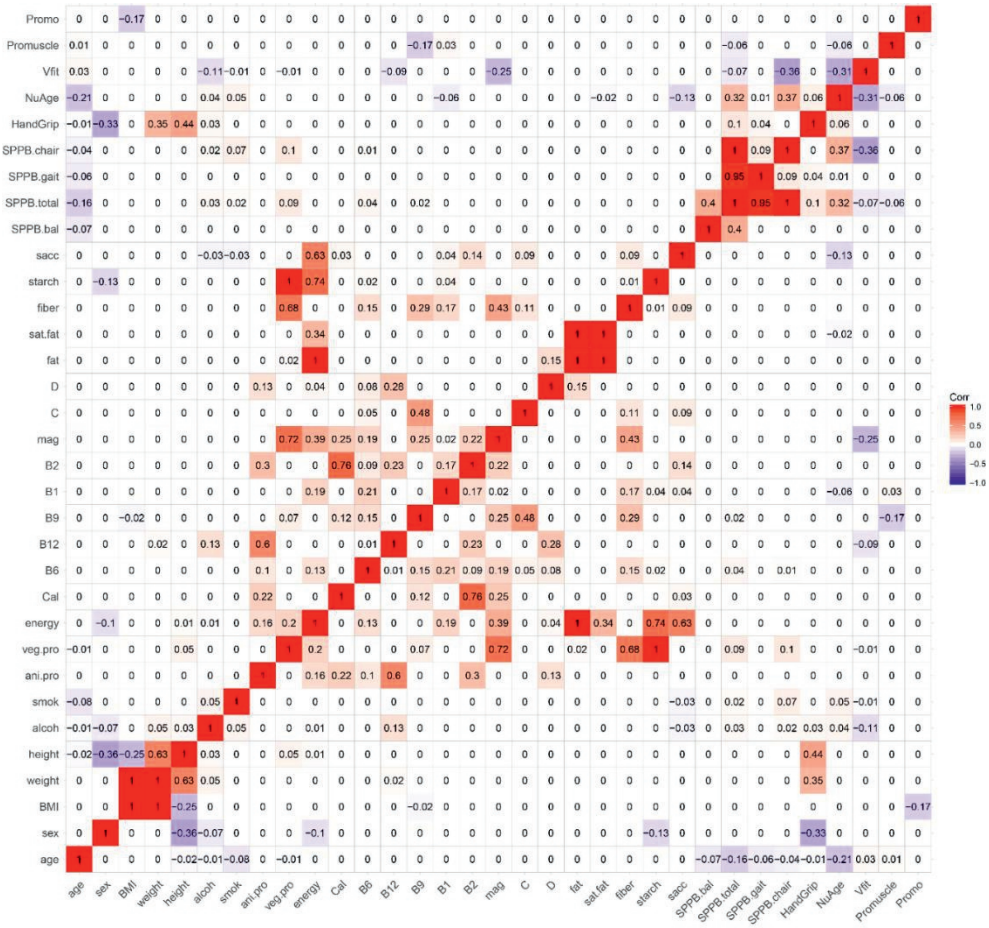
Supplemental Figure 4 Conditional dependencies networks among variables in the ProMO study (*n*=81).

The colour of links depicts positive (blue) and negative (red) partial correlations. The type of line used represents the strength of each edge based on partial correlation values. In this graph, physical functioning items (SPPBs) are isolated from the rest of the networks.

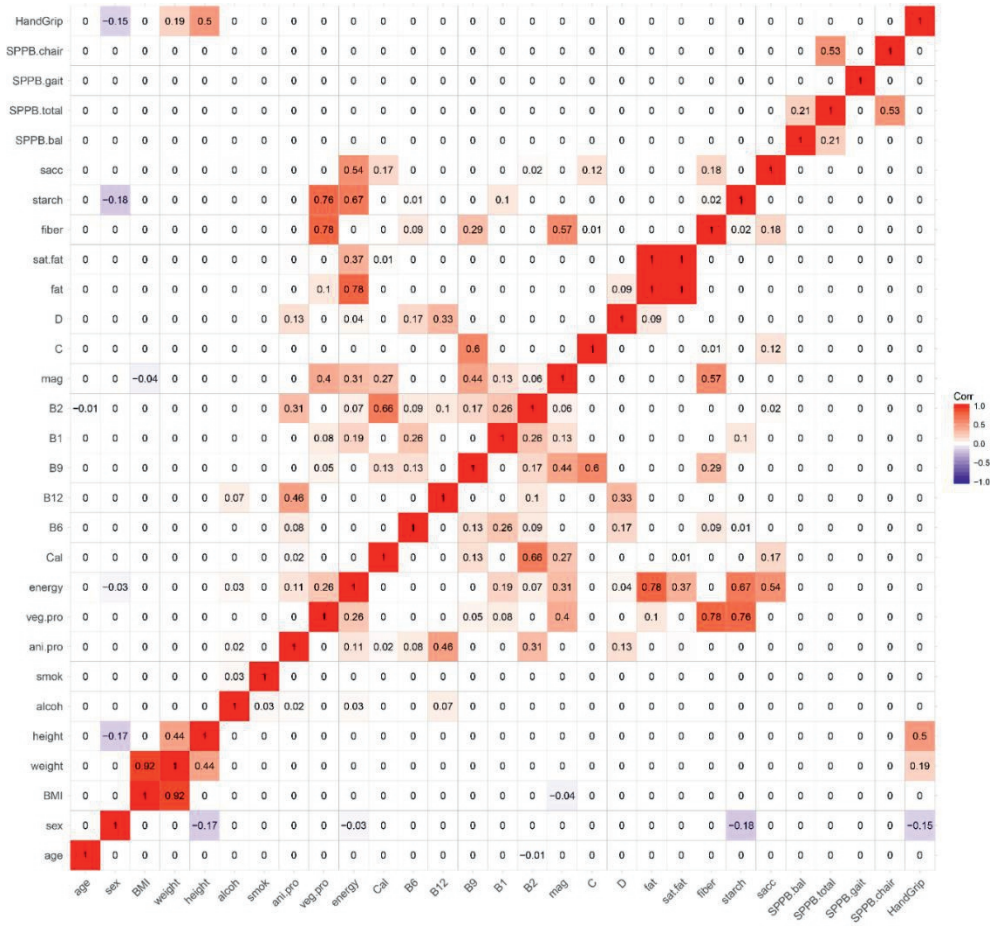


Supplemental Figure 5 Conditional dependencies networks among variables in the V-Fit study (*n*=207).

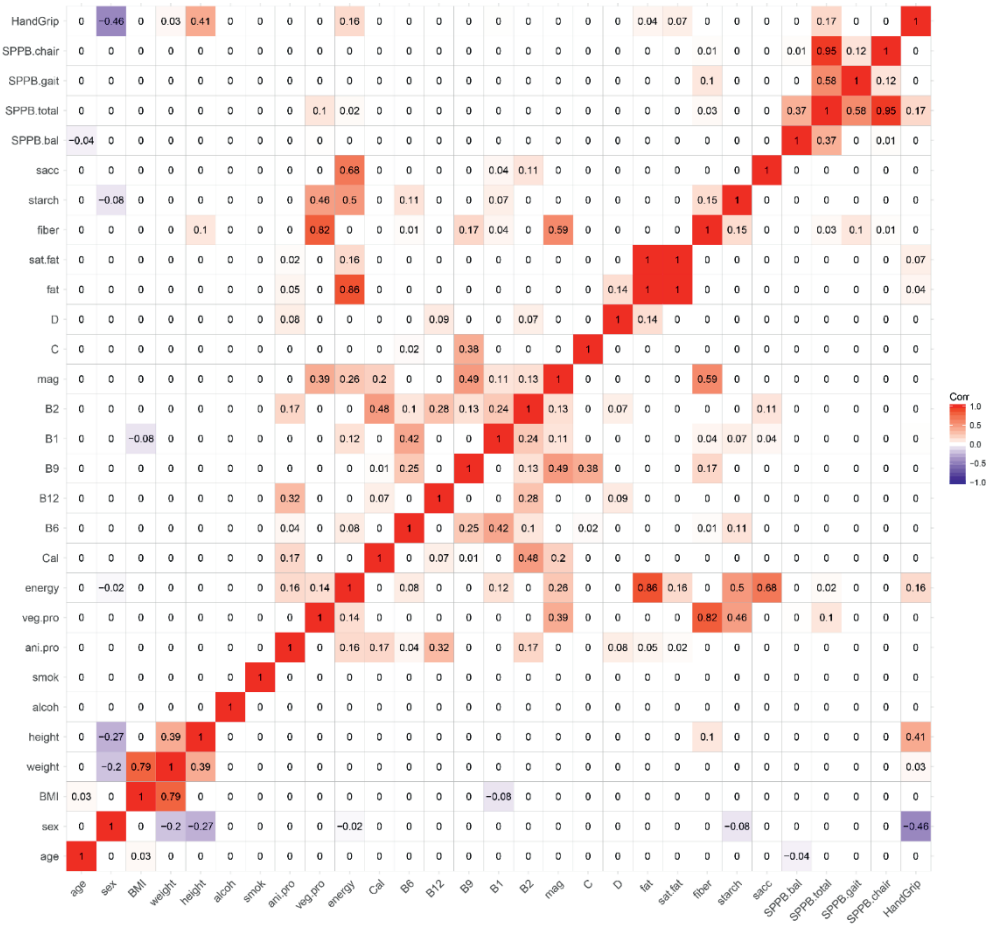
The colour of links depicts positive (blue) and negative (red) partial correlations. The type of line used represents the strength of each edge based on partial correlation values. In this graph, physical functioning items (SPPBs) are isolated from the rest of the networks.



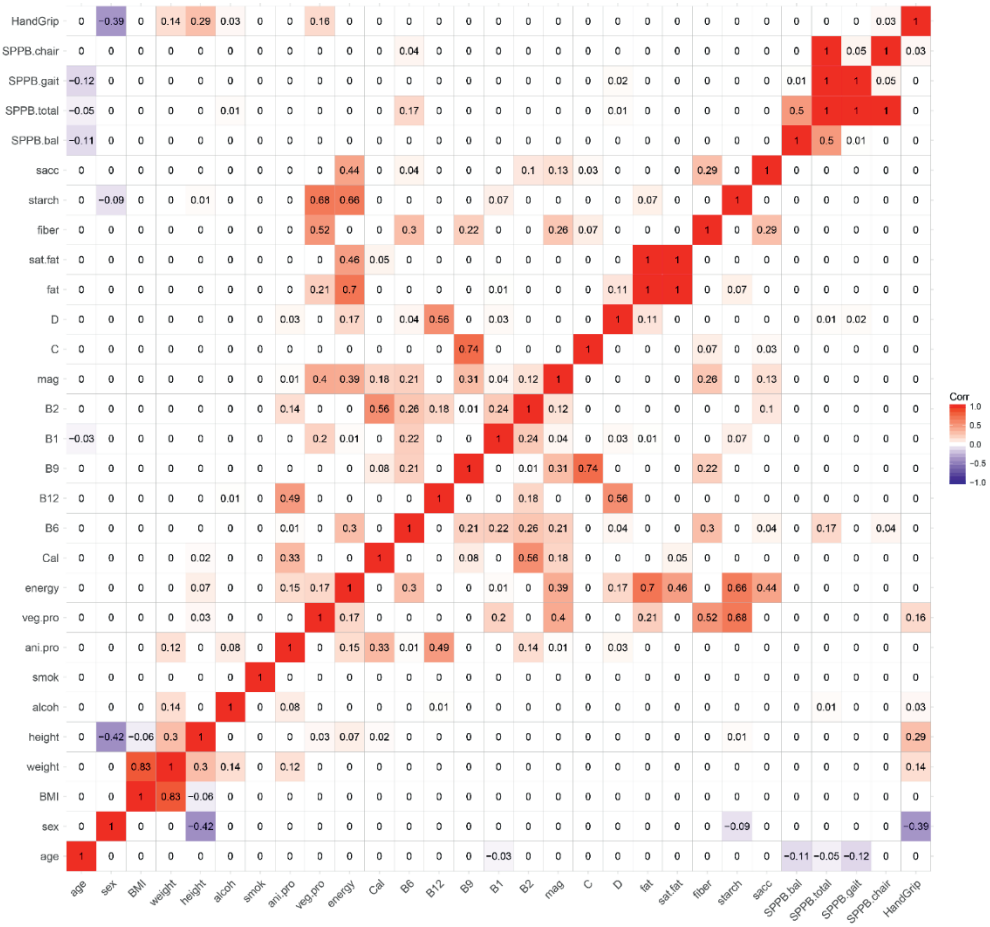
Supplemental Figure 6 The partial correlation coefficients associated with each link in the estimated network for the combined data (*n*=662). Each element represents the partial correlation coefficient between two variables.



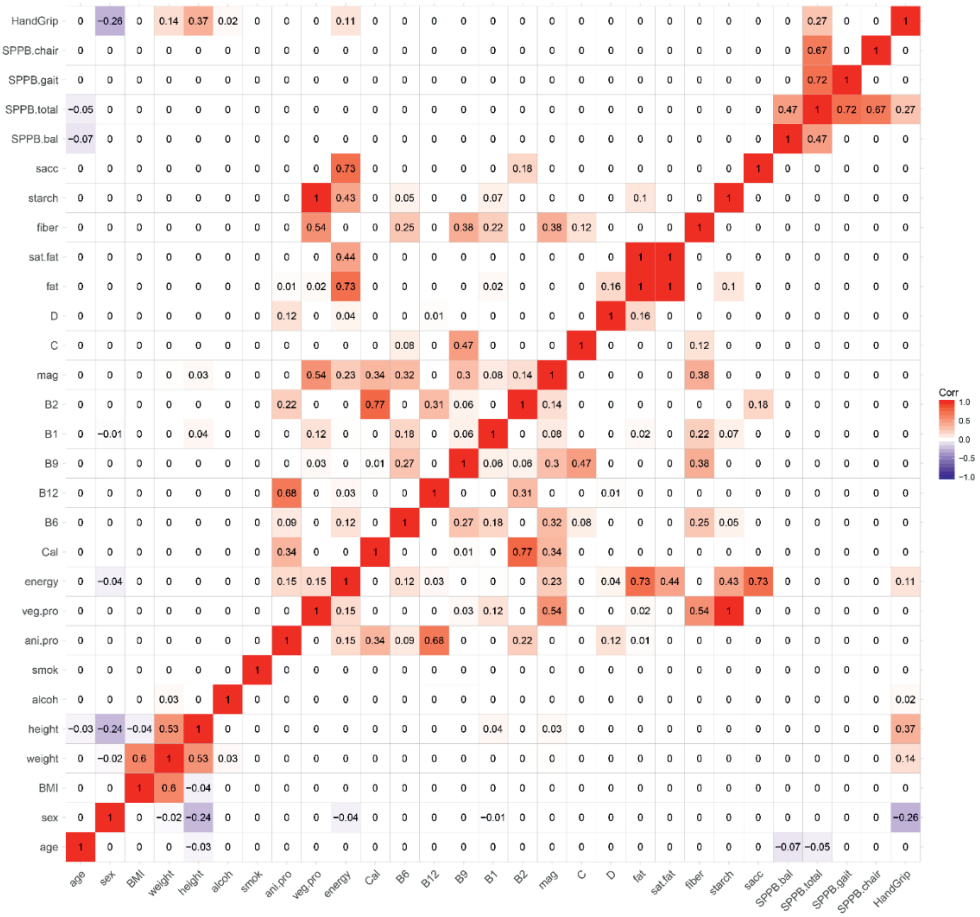
Supplemental Figure 7 The partial correlation coefficients associated with each link in the estimated network for the NuAge study (n=252). Each element represents the partial correlation coefficient between two variables.



Supplemental Figure 8 The partial correlation coefficients associated with each link in the estimated network for the ProMuscle study (n=122). Each element represents the partial correlation coefficient between two variables.



Supplemental Figure 9 The partial correlation coefficients associated with each link in the estimated network for the ProMO study (*n*=81). Each element represents the partial correlation coefficient between two variables.



Supplemental Figure 10 The partial correlation coefficients associated with each link in the estimated network for the V-Fit study (n=207). Each element represents the partial correlation coefficient between two variables.

Fit of model to integrated data

One way to assess the adequacy of the proposed model is to compare the fitted model with the saturated model. Let $l_m(\widehat{\boldsymbol{\Omega}})$ and $l_s(\widehat{\boldsymbol{\Omega}})$ be the log-likelihood of the observations for the fitted model and the saturated model, respectively. The deviance statistics which is defined as

$$D = l_m(\widehat{\boldsymbol{\Omega}}) - l_s(\widehat{\boldsymbol{\Omega}})$$

allows us to assess the goodness-of-fit of the proposed method. Table 1 shows how well the proposed model fits the data. The chi-square test with 401 degrees of freedom gives a p-value of 1, indicating that the proposed model fits the data adequately.

Table 1 A summary of model fit to the integrated data

Model	df	Log-likelihood	Deviance	P-value
Fitted model	128	−4847.58		
Saturated model	528	−1856.94		
Fitted model vs Saturated model	401		5981.28	1

Chapter 7

General Discussion

This thesis aimed to explore nutritional strategies to improve or maintain muscle quality during ageing. Chapter 2 underpins the validity and the reliability of handheld dynamometry to measure lower limb strength in older adults. The main findings, as presented in other chapters of this thesis, are summarised in **Table 1**. A four-week nutrition and exercise program studied in Chapter 3 improved muscle mass and endurance capacity. Endurance performance was also improved via a novel medical nutrition product in Chapter 4. Nutritional strategies to improve muscle strength were identified in Chapter 3, 5 and 6. This thesis successfully identified new intervention strategies with nutrition and exercise to improve muscle quality, defined as the relative functioning of the muscle tissue. This final chapter discusses the main findings of this thesis, its methodological considerations, implications for clinical practice and suggestions for future research. This chapter is divided into six statements and ends with an overall conclusion.

Table 1. Main findings of this thesis.

Outcome	**Muscle mass**		**Muscle strength**		**Endurance capacity**	
Parameter	[A] Quadriceps cross-sectional area [B] Whole body lean mass		[A] Chair rise test [B] Knee extension/flexion strength test [C] Handgrip strength test		[A] Estimated VO2-max [B] Gait speed	
Intervention	Positive effect	No effect	Positive effect	No effect	Positive effect	No effect
4-weeks training program + 30 grams of milk protein per day	Chapter 3: +5%[A]		Chapter 3: +20%[A] +13%[B]	Chapter 3: NS[C]	Chapter 3: +10%[A]	
Novel medical nutrition product with ursolic acid, whey/casein mixture, extra vitamin D and branched-chain amino acids		Chapter 4: NS[B]		Chapter 4: NS[ABC]	Chapter 4: +10%[B]	
Exposure	Positive association	No association	Positive association	No association	Positive association	No association
Vitamin B3				Chapter 5: NS[AC]		Chapter 5: NS[B]
Vitamin B6			Chapter 5: +3% per mg[A] +3% per mg in least active quartile[C] Chapter 6: Direct relationship[A]	Chapter 6: NS[C]		Chapter 5,6: NS[B]
Vitamin B12			Chapter 6: Direct relationship[A]	Chapter 5: NS[A] Chapter 5,6: NS[C]		Chapter 5,6: NS[B]
Folate				Chapter 5,6: NS[AC]		Chapter 5,6: NS[B]
Vegetable protein			Chapter 6: Direct relationship[A]	Chapter 6: NS[C]		Chapter 6: NS[B]

NS, no significant effect; empty cells reflect that the outcome measure was not studied.

Statement 1: Knee extension strength assessment can replace handgrip strength assessment for the monitoring of changes in strength in older adults

Chapter 2 is not included in Table 1 since this chapter does not report on a strategy to improve a muscle quality-related outcome. However, the findings of Chapter 2 are most relevant for the future of muscle quality research, considering the observed usability of handheld dynamometry (HHD) for the assessment of leg strength in research and clinical practice.

Handgrip strength is often measured in ageing research and clinical practice, mostly by dynamometry, using inexpensive, small and light devices that are practically feasible. To signal low muscle strength in older adults, the consensus on sarcopenia diagnosis recommends the assessment of handgrip strength (or chair rise test) [1]. However, handgrip strength assessment is prone to several critical limitations, which are less pronounced in leg strength assessment. First, the evaluation of handgrip strength is highly influenced by arthritis-like conditions [1]. Osteoarthritis is common in older adults[2] and presents itself more often in hands than in knees [3]. Secondly, handgrip strength provides information of lower clinical value compared to leg strength, as it is a worse estimate of future functional decline [4] and general health [5]. Finally, handgrip strength does not reveal changes in strength over time [6]. The ability to assess changes in strength over time is vital for the assessment of the efficacy of interventions aimed at improving muscle strength or muscle quality. Illustrative to the inability of handgrip strength measurements to capture changes in strength over time are the results of Chapter 3, where a 4-week nutrition and exercise intervention was tested. In Chapter 3, substantial improvements in chair rise test and knee extension strength were not reflected in the repeated assessments of handgrip strength.

To be able to shift from handgrip strength assessment to leg strength assessment, clinicians and researchers need a device that can be used in practice, which is an important feature of handgrip strength dynamometry. The golden standard for leg strength assessment is isokinetic dynamometry [7]. This measurement is performed by using static and costly equipment, such as the Biodex Systems 4. The handheld dynamometer (HHD) forms an interesting alternative, as it is inexpensive, portable, and easy to use, for instance, at the patient's bedside. In Chapter 2, the reliability of the HHD, and its validity relative to the golden standard were investigated.

Though the HHD showed excellent relative validity to the Biodex System 4 in Chapter 2, a systematic overestimation of leg strength in HHD measurements as compared to Biodex measurements was observed. The observed validity implies that the HHD measurements are suitable to rank older adults by knee strength. The systematic overestimation infers that the HHD readings should not be compared to strength cut-offs, for instance to diagnose sarcopenia because this will lead to low specificity. The HHD showed excellent intra-rater and interrater reliability, even when measurements were performed eight weeks apart. Such reliability indicates that handheld dynamometry can be used in research and clinical practice to monitor strength over time or to assess the effectiveness of interventions aimed at improving muscle strength or muscle quality.

In Chapter 2, data were collected in older adults who estimated their own strength to be below the average for their age. This inclusion-criterium was prone to different interpretations and resulted in a sample of older adults with a large variation in knee strength (extension force: mean ± SD 345 ± 108 N). In this study, no indications for a strength-related bias were observed. Therefore, the reliability and validity values can be assumed to be similar in populations with lower and higher strength levels. The next step towards further implementation of the HHD in research and clinical practice would be to examine the possibility of calibrating HHD measurements to reduce systematic errors.

Statement 2: A four-week programme of nutrition and exercise already improves muscle mass and strength, and fits well in the current time window for prehabilitation

In Chapter 3, we demonstrated that a nutrition and exercise program of just four weeks already leads to improvements in muscle mass, muscle strength and aerobic capacity. The increase in muscle strength (+13%) was larger than the increases in muscle mass (+5%). Such changes counteract the process of muscle ageing, as described in Chapter 1, during which muscle strength decreases faster than muscle mass does. The larger increase in muscle strength compared to muscle mass indicates an enhanced muscle quality. Indeed, in the dominant leg, we observed an improvement in muscle quality, expressed as torque per unit of muscle cross-sectional area. The finding that it is possible to improve muscle mass and strength in older adults already within four weeks is of great importance for the field of prehabilitation. Interventions that showed to be effective in improving muscle mass and strength so far achieved that in around four months [8, 9]. Prehabilitation aims to improve muscle mass and strength in the pre-surgical period, which is usually around 30 days [10, 11].

To gauge the clinical relevance of the improvements in muscle mass before surgery, they can be weighed against the inevitable loss of muscle mass during hospitalisation. In Chapter 3, we found an increase of 5.4% in quadriceps cross-sectional area, while a 3.4% decrease in quadriceps cross-sectional area during a week of hospitalisation has been reported [12]. When combining these data in one plot (**Figure 1**), the net preservation of muscle mass during hospitalisation is clearly visible. The crude data in the figure suggest even a net positive result in muscle mass achieved by prehabilitation, but further research should quantify the significance of that result. Furthermore, the line in this figure reflects the loss during one week of hospitalisation and does not include post-discharge muscle atrophy, which is likely to occur in real life [13]. It is therefore important to stress that prehabilitation alone might be inadequate in the long run, and postoperative strategies should also be part of a comprehensive perioperative strategy. After hospitalisation, the potential of nutrition and exercise to maintain and improve muscle mass could be exploited, for instance via anabolic nutrients like protein [14], leucine [15], β-hydroxy β-methyl butyric acid [16], and via exercise or exercise mimetics within the abilities of the patient [2].

Chapter 3 was set-up as a pilot trial, therefore consisting of only one research arm. The results should, therefore, be interpreted with some caution. Measurements like the chair rise test and the knee extension strength test are prone to a learning effect, which might partially explain the improvements over time. On the other hand, the quadriceps cross-sectional area was measured by MRI and is not sensitive to learning effects. Also, the VO2-max assessment was performed by a heart rate based Åstrand test, which is supposedly less affected by learning effects because it is mainly influenced by physiological values instead of mental determinants. The training program in Chapter 3 was investigated in a sample of healthy older adults, not in patients awaiting planned surgery. Although this sample of older healthy adults gives us valuable information on possible improvements within a short time-frame, results might be different in populations that are actually planned for surgery [17, 18]. Therefore the preferred design for a future study would be to investigate a combination of the prehabilitation program of Chapter 3 and postoperative care aimed at muscle mass maintenance, in a randomised controlled trial amongst older adults that do undergo planned surgery.

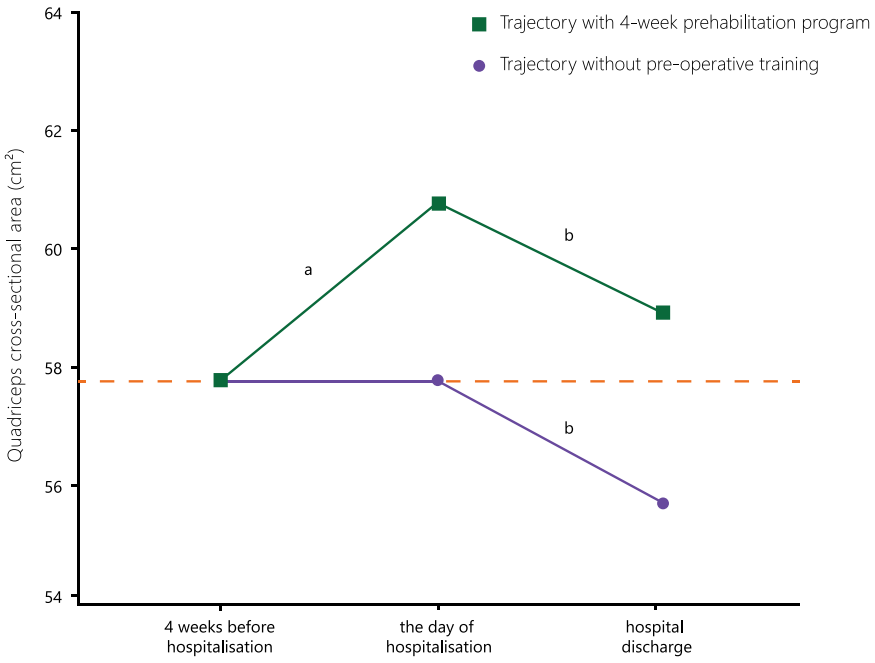


Figure 1. The projected trajectory of the potential of prehabilitation to prevent net losses in muscle mass in older adults during hospitalisation, after plotting the increase found in Chapter 3 (a, +5.4%) and the decrease adapted from Kouw et al. [12] (b, -3.4%). The orange dotted line represents the starting level of the quadriceps cross-sectional area.

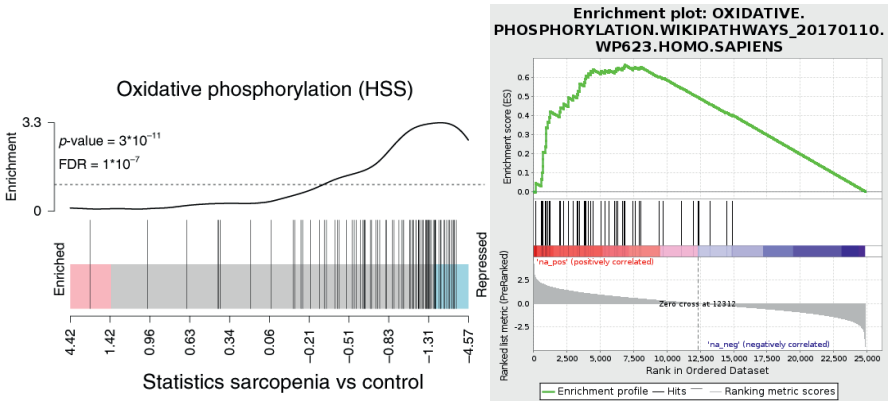


Figure 2. The repression of oxidative phosphorylation that is observed in sarcopenic older adults as shown by Migliavacca et al. [19] (left panel) can possibly be reverted by the novel nutritional intervention as used in the study described in Chapter 4 (right panel).

Statement 3: Nutritional strategies can improve physical performance even in the absence of concurrent exercise training

In Chapter 5, the associations between intake of B-vitamins and physical functioning were investigated. Dietary intake of vitamin B6 was associated with greater handgrip strength (+3% per mg increase in vitamin B6 intake), but only in the participants in the lowest quartile of physical activity. The median activity level of these participants, expressed as PASE-score, was only 72, where a PASE-score of at least 130 is recommended for older adults [20]. To put this number into perspective: increasing the PASE-score from 72 to 130 requires at least two additional hours of moderate to strenuous physical activity on at least five days per week [20]. These participants were thus not only highly sedentary relative to the rest of this specific study population, but also relative to current reference values.

The observation that dietary intake of vitamin B6 was only associated with greater handgrip strength in these inactive, otherwise healthy, older adults, and not in those with a higher activity level, implies that physical activity modifies the relationship between diet and physical performance. Indeed, high physical activity levels can result in lower plasma levels of vitamin B6 [21], speculatively because vitamin B6 is needed for amino acid metabolism and glucose breakdown [22]. Still, not much is known about how physical activity could mechanistically influence relationships of nutrients like vitamin B6 and physical performance in older adults. To further unravel these complex relationships, employing copula graphical modelling (as used in Chapter 6) on large datasets of older adults with varying activity levels could be a next step. It is important that such datasets include complete and accurate data on dietary intake, physical activity and physical performance.

Chapter 4, which investigated a novel medical nutrition product, is another example of the potential of nutrition in older adults with a low habitual physical activity level. The participants in this study were sedentary at baseline, and their level of physical activity, which was measured via accelerometry, did not increase during the study. In this study, an improvement in muscle strength was found in participants using a standard oral nutritional supplement (+12%), but only for one out of four strength tests. On the other hand, in participants who used a novel formulation of oral supplementation, walking performance improved (+10%), on both a long distance and a short distance. Such improvements in walking performance are relevant for older adults, as they predict a lower risk of future falls, hospitalisation and mortality [23, 24]. Molecular analyses on skeletal muscle tissue suggested that the improved

gait speed was a result of improvements in mitochondrial functioning and upregulation of oxidative phosphorylation. Because participants did not increase their physical activity level, the results of Chapter 4 indicate that physical performance can be improved via nutrition alone, in the absence of concurrent exercise.

Chapter 4 was performed in a strict sample of older adults with (risk of) undernutrition. As this sample is highly distinct, careful generalisation of the results of this study to other populations of older adults is warranted. Interestingly, there is an overlap between vulnerability phenotypes in older adults [25]. In Chapter 4, 43% of the undernourished participants were also sarcopenic, indicating possible broader generalisability of results. Interestingly, recently Migliavacca et al. [19] compared genome-wide differences in transcriptomics in sarcopenic older adults compared to age-matched controls. Their main finding was that the sarcopenic older adults showed clear repression of gene-sets related to mitochondrial functioning and oxidative phosphorylation. Strikingly, the gene sets that were upregulated by the novel oral nutritional supplement in Chapter 4 show an upregulation of the exact same gene sets (illustrated by the enrichment in the oxidative phosphorylation gene set in **Figure 2**). The finding that a nutritional treatment can revert the repression of sarcopenia-related gene sets in a malnourished population with a high prevalence of sarcopenia, suggests that nutrition might be able to slow down the sarcopenic process, at least partly and at least at the level of the transcriptome.

The current standard treatment against sarcopenia is resistance exercise training [26]. Indeed, resistance exercise shows beneficial effects on muscle mass and muscle strength in older adults [8, 9]. Meta-analyses performed on studies in healthy older adults concluded no clear additional effects of nutritional support [27, 28], but a meta-analysis of the few available randomised controlled trials in sarcopenic older adults did suggest a role for nutritional support in this population [29]. Importantly, long-term adherence to exercise regimens is often low [30, 31], and limitations in health, physical functioning or mental state can hamper the applicability of resistance exercise, while nutritional interventions might still be feasible. All in all, given the current evidence, it is warranted to focus on exercise interventions in the first place, but the potential of nutrition to improve physical performance during ageing should not be neglected.

Statement 4: Complex network analyses enrich the field of nutritional epidemiology

Adjusting for confounding variables is a key practice in nutritional epidemiology. However, current approaches are often criticised [32, 33]. Of great concern is the way researchers decide on which confounders to adjust for in their statistical models. Confounders are chosen based on subjective factors, such as the study design, the background knowledge of the researcher and even on the sample size of the study [34]. Recently, two papers called out to authors to limit the use of old-fashioned, subjective methods and to editors and reviewers to appreciate new, less subjective methodology [32, 33]. Such a new and less subjective methodology could be Copula Graphical Modelling (CGM), as used in Chapter 6 of this thesis.

Graphical modelling is extensively implemented in fields with large, multi-level datasets, such as genetics [35-40] and metabolomics [41, 42]. Also, the field of nutritional epidemiology makes use of large datasets with extensive information on nutritional intake. Intake levels of nutrients are typically intercorrelated and not-normally distributed [33]. Advances in nutrition science have resulted in even larger, multi-level datasets with the inclusion of transcriptomics and metabolomics [43]. With the extension of graphical models to copula graphical models, this type of network analysis is now able to handle ordinal data and not-normally distributed data [44]. This extension makes CGM a suitable statistical method to identify relationships in such datasets, without the need for subjective selection of confounders. Hereby, CGM has many potential applications in future nutritional epidemiology. Only two studies have used graphical modelling in nutrition research before, as to identify dietary patterns [45, 46]. Chapter 6 provides an example of how GCM can be used to study complex relationships between nutrient intake and health outcomes. In that chapter, GCM identified that dietary intake of vitamin B6, B12, folate and vegetable protein is related to improved physical performance in older adults.

The ability to study relationships between nutrient intake and health outcomes makes CGM an exciting tool to further implement in nutritional epidemiology. However, two major concerns should be noted. First, the use of CGM circumvents a lot of subjectivity, but not all. After all, the model is limited by the completeness and quality of the data. The subjective decisions that determine the variables in the dataset, mainly made in the study design phase, cannot be circumvented by the CGM approach. Second, users of this method should be aware of its binary nature. CGM

identifies the presence or the absence of direct correlations. The model will provide information on the direction of the relationship, and on the certainty of the relationship as estimated by bootstrapping. It does, however, not inform on the magnitude of the effect, information that in traditional nutritional epidemiology modelling is retrievable in the *beta*. The second concern relates to the applicability of CGM for different purposes. Using CGM will test all relationships between exposure and outcome in the dataset. Therefore, CGM offers an ideal solution for explorative research. Here, CGMs can be employed as a first step by identifying new targets to address an outcome. In potential next steps, traditional nutritional epidemiology can be used to estimate the magnitude of the relationships, to assess clinical relevance, and ultimately to work towards public health recommendations. Otherwise, if determining causality is the aim, studies investigating the mechanism, or controlled trials can be performed as a second step.

Statement 5: More research towards vegetable proteins for the ageing population is urgently needed given its potential for health and sustainability

In Chapter 6, copula graphical models (CGMs) were applied with the aim to identify new nutritional targets to combat age-related declines in physical functioning. The data from baseline measurements from four intervention trials were jointly analysed. These trials all included Dutch adults aged 65 years and older, but samples were drawn from different populations. The trials included: healthy participants with elevated homocysteine levels [47], (pre-)frail participants [48, 49] and/or undernourished participants [50]. The inclusion of only baseline data results in the absence of information on the temporal order of events. Thus, no conclusions on causation can be drawn from this chapter. Nonetheless, Chapter 6 identified interesting new targets to address physical functioning in older adults.

The most striking finding of this chapter was that dietary intake of vegetable protein was directly correlated to chair rise test performance and to the overall score on the short physical performance battery (SPPB). On the other hand, and against expectations, no direct correlation between the intake of animal protein and any measure of physical performance was found. Based on current evidence, a superior role of animal protein over vegetable protein is assumed. Animal protein attenuates losses in muscle strength during 6 to 9 years of follow-up [51, 52], and leads to a greater increase in muscle protein synthesis as compared to vegetable protein [53]. On the other hand, the evidence for a role for vegetable protein in physical functioning during ageing is scarce [54, 55]. An important notion for cross-sectional studies, like Chapter 6, is that vegetable protein intake is related to overall diet quality. However, the graphical models did adjust for all nutrients, including vitamins and dietary fibre. Still, residual confounding by diet quality could be present.

The main argument for animal protein superiority over vegetable protein is its greater stimulation of muscle protein synthesis [56]. Interestingly, this argument is based on studies comparing animal protein sources with wheat [57] or soy-protein [58-62]. Wheat and soy protein are limited in the amino acids lysine and methionine, respectively, compared to animal-based protein [63]. Future research should compare the muscle protein synthesis response of animal protein sources with non-animal protein sources (or blends) of similar amino acid profiles. Supposedly, even with similar amino acid profiles, the muscle protein synthesis responses after consumption of vegetable proteins will be lower. Vegetable protein has a lower bioavailability compared to animal protein [64], which is mainly caused by differences

in digestibility [65], partly related to the presence of antinutrients in the matrix [66]. Further unravelling of these differences may lead to new bio-availability limiting characteristics of vegetable protein, for which elimination strategies can be explored, such as combining specific protein sources and modifying their matrix. More research towards vegetable proteins for the ageing population is warranted given the potential for health and sustainability [67].

Apart from the identification of a possible prominent role for vegetable protein in physical functioning maintenance during ageing, the CGMs furthermore identified a relationship between intake of vitamin B6, B12 and folate with physical functioning. A role for vitamin B6 in physical functioning was also found in Chapter 5. Vitamin B6, B12 and folate are known for their role in the one-carbon pathway, via which they lower plasma homocysteine concentrations. Homocysteine concentrations are negatively correlated to physical performance [68-70] and homocysteine can impair the function of mitochondria [71-73] and endothelial cells [74-76]. Apart from homocysteine-lowering characteristics, vitamin B6 intake also influences the metabolism of multiple amino acids, neurotransmitters and fatty acids [77]. Vitamin B6 status correlates with plasma concentrations of n-3 fatty acids [78] and creatine [79], which are both related to physical performance [80, 81]. Future research should establish causality between intake of vitamin B6, B12, folate and physical performance.

Statement 6: Clinical trials in older adults that aim to improve physical performance or body composition should include the assessment of muscle quality

Chapter 1 describes how clinical trials so far succeeded in improving the determinants of muscle quality. Different types of exercise regimens seem to be able to increase type II fibre size [82-94], improve mitochondrial [93, 95-102] and neuromuscular functioning [103-112], and lower intramuscular adipose tissue [109, 113-119] in older adults. Interventions with nutrition and bio-actives are able to improve mitochondrial functioning (iron [120], resveratrol [121, 122], nitrate [123] and epicatechins [124]), intramuscular adipose tissue (combined supplementation with whey protein and vitamin D [118, 125]) and neuromuscular activation (N-3 poly-unsaturated fatty acids [126], milk fat globule membrane [127] and creatine [128]). On the other hand, interventions with nutrition and bio-actives seem ineffective in improving type II fibre size [84, 92, 94, 129-143]. The studies performed in this thesis expand the findings of Chapter 1, by showing that markers of muscle quality can be improved via 4-weeks of intense exercise and 30 grams of milk protein per day (Chapter 3), and by a medical nutrition product with free branched-chain amino acids, ursolic acids, whey protein and vitamin D (Chapter 4). Chapters 5 and 6 uncovered possible roles for B-vitamins and vegetable protein in targeting muscle quality in older adults.

A variety of nutrients and bio-actives seem to be effective in improving muscle quality. Clinical trials investigating nutrients and bio-actives to target different aspects of muscle quality are still scarce. More research into improving muscle quality via nutrients or bio-actives is therefore warranted, as it is likely that new effective nutritional strategies can be found. Yet, more importantly, muscle quality assessment should more often be incorporated in clinical trials with nutrition amongst older adults. Experiments that investigate outcomes related to muscle mass should seriously consider at least the addition of handheld dynamometry (HHD) to their assessment battery. Chapter 2 shows that HHD is able to reliably assess changes in leg strength over time. Combining these strength readings with muscle mass assessments yields the possibility to calculate muscle quality as relative strength (strength divided by a unit of mass or volume), and thereby, assessing changes in muscle quality over time.

The assessment of muscle quality can be extended by measurements of the determinants of muscle quality. Assessing type II fibre size is only recommended in

case of a resistance exercise intervention, as nutrients and bio-actives do not seem effective for this purpose. Mitochondrial functioning can be assessed via transcriptomics on skeletal muscle tissue acquired via micro-biopsies or via a ^{31}Phoshorus nuclear magnetic resonance phosphocreatine recovery protocol [144]. Less invasively, mitochondrial functioning can be estimated with near-infrared spectrometry combined with limb occlusions [145]. Intramuscular adipose tissue can be measured non-invasively via ultrasound [146]. Voxel intensity of acquired ultrasound images of skeletal muscle tissue provides a valid estimate of intramuscular adipose tissue. Neuromuscular activation can be measured non-invasively via electromyography [147].

Increased inclusion of muscle quality assessment in clinical trials aimed at improving physical functioning in older adults will increase the understanding of the role of nutrition in improving or preventing age-related declines in muscle quality. Ultimately, findings of such clinical trials can be translated into public health care recommendations to improve the level of physical performance in older adults. With improved physical performance, older adults are also more likely to stay mentally active, socially active and physically active, which helps them to age healthily and happily.

Conclusions

To conclude, there are several ways to improve muscle quality via nutrition, bio-actives, exercise regimens, or a combination of these. Based on the findings of this thesis, the following can be concluded: (1) changes in muscle strength can be assessed reliably in research and in clinical practice via handheld dynamometry, (2) improvements in muscle quality can be obtained within four weeks with intensive concurrent exercise training combined with protein supplementation, (3) including whey protein, free branched-chain amino acids, ursolic acid and extra vitamin D the formulation of medical nutrition can improve mitochondrial functioning, and (4) dietary intake of vegetable protein, vitamin B6, B12 and folate may play roles in the maintenance of physical performance during ageing. The work in this thesis adds important knowledge to the field of nutrition and ageing research. Advancing the field of nutrition and ageing research can result in increased independence and quality of life of older adults and ultimately in improved health and social participation.

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Summary

Summary

Increased lifespan is a great achievement of humankind, and opens new opportunities for the life of ageing human beings, in part conditional on health and independence. Health and independence are related to physical performance which is often compromised in older adults, to some extent due to age-related decreases in muscle mass and muscle strength decrease. During ageing, muscle strength decreases at a faster rate than muscle mass, indicating a loss in muscle quality. Muscle quality is defined as muscle strength per unit of muscle mass and is determined by factors such as specific muscle fibre atrophy, mitochondrial functioning, neuromuscular functioning and degree of fat infiltration. Targeting the quality of muscle tissue is a promising strategy to improve muscle quality during ageing.

An active lifestyle seems to prevent losses in muscle quality to some extent, whereby exercise programs can improve markers of muscle quality. Not many intervention trials have aimed to improve muscle quality via nutrition. However, metabolic aspects play a role in muscle quality, and many nutrients act on pathways that are related to physiological determinants of muscle quality. Together, this gives reasons to pursue the identification of nutritional strategies to target muscle quality. Therefore, in this thesis, we aimed to identify novel nutritional strategies to improve muscle quality and physical performance in older adults.

In Chapter 2, we investigated the reliability and validity of handheld dynamometry (HHD) for the assessment of leg strength. Leg strength assessment is proposed to be a better alternative to the often used handgrip strength measurement. However, the current advised equipment to measure leg strength is static and costly, while handgrip strength can be measured with an inexpensive and small device. The results of Chapter 2 show that the HHD is a valid and reliable tool to assess changes in leg strength in older adults or to rank older adults on strength level, indicating that HHD can be used as an alternative to handgrip strength assessment. The results of Chapter 2 are important for the development of leg strength measurements for strength monitoring in older adults.

Chapter 3 and Chapter 4 describe the results of two clinical trials aimed at improving physical performance in older adults. In Chapter 3, an intensive exercise and nutrition intervention augmented muscle mass (+5%), strength (+13%), and physical performance (10-20%) of older adults already after four weeks. The findings are relevant and important because short-term interventions like these are urgently needed for the future of prehabilitation.

Prehabilitation is a discipline aimed at training patients before planned surgery so that patients lose less muscle mass and strength during hospitalisation.

In Chapter 4, a novel medical nutrition product was tested against standard medical nutrition in older adults with (or at risk of) undernutrition. Without a concurrent exercise regimen, this novel medical nutrition treatment appeared to be able to improve walking performance and mitochondrial functioning in this vulnerable group. The improvement in walking performance is of clinical relevance, as it relates to lower chances of future falls, hospitalisation and mortality. Being able to improve physical performance without a concurrent exercise program is an important finding for the field of nutrition and ageing, suggesting that besides exercise interventions, there is a potential for nutritional interventions as well.

In Chapter 5 and Chapter 6, we aimed to identify new nutritional targets to improve muscle quality in older adults. Chapter 5 was a hypothesis-driven approach in which we tested the associations between dietary intake of vitamin B3, B6, B12, folate and measures of physical performance in healthy older adults. Chapter 6 was a data-driven approach, in which we tested for associations between all nutrients and measures of physical performance in healthy, frail or malnourished older adults. Chapter 5 identified an association between the intake of vitamin B6 and chair rise test in the full population (3% improvement per mg of vitamin B6) and an association between intake of vitamin B6 and handgrip strength in participants with low physical activity (also a 3% improvement per mg of vitamin B6), but not in those with normal or high physical activity. Chapter 6 also identified an association between vitamin B6 and chair rise test and total physical performance score. Chapter 6 additionally identified associations between intakes of vegetable protein, vitamin B12 and folate with measures of physical performance. Chapter 6 made use of Copula Graphical Modelling, a new methodology with many potential applications for the field of nutritional epidemiology.

This thesis adds important knowledge on how to measure muscle quality, which strategies are effective and which nutritional strategies could be further explored in the future. Advancing the integration of muscle quality measurements in the field of nutrition and ageing research can lead to important advances in the quest to find strategies that improve physical performance in older adults. With improved physical performance, older adults are more likely to stay mentally, socially, and physically active. This in turn helps them to age healthily and happily.

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About the author

Curriculum vitae

On Sunday the 16th of July, 1989, in the small Northern-Brabant village of Sprang-Capelle, Pol Grootswagers was born as the third child in a family of four. After completing secondary school at the Dr Mollercollege in Waalwijk in 2007, Pol earned his propaedeutical diploma in Biology Teaching (Fontys Tilburg, 2019) and his BSc degree in Nutrition and Dietetics (HAN Nijmegen, 2013). In 2014, he enrolled in the MSc program Nutrition and Health at Wageningen University. He completed his MSc thesis in the field of nutrition and ageing and performed his internship at TNO, where he studied personalised nutrition. After graduating in 2016, Pol started his PhD on nutrition and ageing, with a particular focus on muscle mass and muscle quality.

During his PhD, Pol coordinated a large clinical trial with older malnourished participants. He presented the results of this project during international conferences in Italy, Spain, Sweden, Canada and during many national meetings. He also worked together with the statistical department of Biometris, was involved in the TiFN framework Mitochondrial Health and completed a project in the Eat2Move consortium. He was chairperson of the organisational board of the 2019 PhD tour to Canada, member of the Governance Table of the division between 2018 and 2020, and involved as a teacher in the Massive Open Online Course (MOOC) Nutrition, Exercise and Sports, and in various Bachelor and Master courses. He supervised ten MSc thesis students and five BSc thesis students. He obtained a seed grant by the ProteinTransition community, which he used to write a grant proposal on alternative proteins for healthy ageing.

In April 2020, he started as a postdoctoral researcher in the lab of Lisette de Groot, where he will continue the investigation into nutritional strategies to support healthy ageing.

List of publications

Publications

Grootswagers P, de Regt M, Domić J, Dronkers J, Visser M, Witteman B, Hopman M, Mensink M (2020) A 4-week exercise and protein program improves muscle mass and physical functioning in older adults - A pilot study, *Experimental Gerontology* 141:111094. DOI: 10.1016/j.exger.2020.111094

Behrouzi P, **Grootswagers P**, Keizer PLC, Smeets ETHC, Feskens EJM, de Groot CPGM, van Eeuwijk FA (2020) Dietary Intakes of Vegetable Protein, Folate, and Vitamins B-6 and B-12 Are Partially Correlated with Physical Functioning of Dutch Older Adults Using Copula Graphical Models, *Journal of Nutrition* 150(3), 634-643. DOI: 10.1093/jn/nxz269

Grootswagers P, Vaes AMM, Tieland M, De Groot CPGM (2019) Calcifediol supplementation to reduce pulse pressure in a limited sample of vitamin D deficient older adults with elevated parathyroid hormone levels, *Clinical Nutrition Experimental* (24), 77-82. DOI: 10.1016/j.yclnex.2019.01.003

Submitted manuscripts for publication

Grootswagers P, Vaes AMM, Hangelbroek R, Tieland M, Van Loon LJC, De Groot CPGM. Relative validity and reliability of isometric lower extremity strength assessment by using a hand-held dynamometer in older adults

Grootswagers P, Smeets ETHC, Oteng AB, De Groot CPGM. A novel oral nutritional supplement improves gait speed compared to standard supplement in older adults with (or at risk of) undernutrition: results from a randomised controlled trial

Grootswagers P, Mensink M, Berendsen AAM, Deen CPJ, Kema IP, Bakker SJL, Santoro A, Franceschi C, Meunier N, Malpuech Brugère C, Bialecka-Debek A, Rolf K, Fairweather-Tait S, Jennings A, Feskens EJM, De Groot CPGM. Vitamin B-6 intake is related to physical performance in European older adults - the NU-AGE study

Overview of completed training activities

Discipline specific activities	Organiser and location	Year
Courses		
Modelling of habitual dietary intake	VLAG, Wageningen, NL	2017
Energy metabolism and body composition – *oral presentation*	VLAG, Wageningen, NL	2018
Stable Isotope Methods in Nutrition Research	VLAG, Wageningen, NL	2019
Conferences and meetings		
Food for Thought	Alliantie Voeding en Gezondheid, Ede, NL	2016-2020
10th International Congress on Sarcopenia, Malnutrition and Wasting Disorders – *oral presentation*	SCWD, Rome, IT	2017
Vrijdaglunch Lecture – *oral presentation*	Ziekenhuis Gelderse Vallei, Ede, NL	2018
40th ESPEN Congress on Clinical Nutrition & Metabolism– *oral presentation*	ESPEN, Madrid, ES	2018
TiFN Retreat	TiFN, Arnhem, NL	2018
Opleidingsdag geriators – *oral presentation*	RadboudUMC, Ede, NL	2018
Dutch Nutritional Science Days – *two oral presentations*	NAV, Heeze, NL	2018 & 2019
The Dutch Society for Research on Ageing – annual meeting	DUSRA, Leiden, NL	2018 & 2019
International Association of Gerontology and Geriatrics European Region Congress 2019 – *oral presentation*	IAGG-ER, Gothenburg, SE	2019
VoedingNL congres Eiwit en Spiermassa– *poster presentation*	PitActief, Utrecht, NL	2019
Nationaal Voedingcongres – *oral presentation*	NESPEN, Veenendaal, NL	2019
Symposium Praktijkonderzoek in de kijker – *oral presentation*	HAN, Nijmegen, NL	2019
Highlights IAGG-ER Congress – *oral presentation*	Friesland Campina, Wageningen, NL	2019
Pioneering Nutrition Symposium – 50th-anniversary division of Human Nutrition – *oral presentation*	HNH, Wageningen, NL	2019
TiFN Retreat – *poster presentation*	TiFN, Hilvarenbeek, NL	2019
Lecture Nutrition and Ageing – *oral presentation*	Friesland Campina, Wageningen, NL	2019
Guest lectures Scientific Research – *two oral presentations*	HAN, Nijmegen, NL	2019
International Conference on Frailty and Sarcopenia Research – *two oral presentations*	ICFSR, Toulouse, FR	2020

General courses and activities		
VLAG PhD week	VLAG, Wageningen, NL	2017
Symposium Publish For Impact	WUR Library, Wageningen, NL	2017
Master class Mixed Models	Leiden University, Wageningen, NL	2017
PhD Carousel	WGS, Wageningen, NL	2018
Career perspectives	WGS, Wageningen, NL	2018
Scientific writing	Wageningen in'to languages, Wageningen, NL	2019
Pitch training	Rick Koster, Wageningen, NL	2019
Optional courses and activities		
Monthly Muscle meeting	WUR, Wageningen, NL	2016-2019
Preparation of research proposal	WUR, Wageningen, NL	2017
PhD study tour to UK	WUR, UK	2017
Quarterly TiFN Expert meeting	TiFN, Wageningen, NL	2017-2020
Biweekly Energy Metabolism meeting	WUR, Wageningen, NL	2017-2020
Newtrition board member	WUR, Wageningen, NL	2017-2019
PhD study tour to Canada chairman	WUR, NL and CA	2018-2019
HNH Governance Table member	WUR, Wageningen, NL	2018-2020

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