CAUSES OF UNDERNUTRITION AND ITS CONSEQUENCE FOR PHYSICAL FUNCTION IN OLDER PERSONS

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Understanding how to maintain independence and prevent disability in older persons represents a priority in aging research. Although undernutrition is considered as a putative and potentially modifiable factor contributing to decline in physical function with aging, there is a lack of empirical evidence of its effect on consequent decline in muscle strength and physical function. Furthermore, whether physiological factors such as ghrelin, leptin and inflammatory markers may affect dietary intake in older persons is unknown. The aim of this dissertation was to address this gap in knowledge by examining the following hypotheses: 1) a low intake of protein is associated with subsequent reduction in muscle strength; 2) a low concentration of nutrients is associated with subsequent decline in physical function; 3) concentrations of ghrelin, leptin, and inflammatory markers are associated with reduced dietary intake. To test these hypotheses, we used a population-based, NIH-funded epidemiological study involving over 1100 persons aged $\geq$65 years living in Tuscany, Italy.

We found that selectively in participants with high levels of C-reactive protein, Interleukin-6 and Tumor Necrosis Factor-α, lower protein intake was associated with a greater decline in muscle strength (Beta for CRP=0.020, p=0.003). Furthermore, we demonstrated that a low concentration of vitamin E was significantly associated with subsequent decline in physical function (odds ratio=1.62; 95% confidence interval=1.11-2.36; p=0.01). A classification and regression tree analysis showed that among persons aged 70 to 80 years, the strongest predictor of decline in physical

function was a vitamin E concentration < 32 umol/L (p=60%). Finally, we found that a high concentration of leptin was significantly associated with accelerated decline in energy intake (Beta=-38.0 kcal; p=0.039), and the effect of inflammatory markers on energy intake depended on sex (Beta for CRP(log)*sex=70.0, p=.020).

This is the first population-based, longitudinal study on the effect of poor nutrition on decline in muscle strength and physical function, and on the effect of ghrelin, leptin, and inflammatory markers on dietary intake. This study provides empirical evidence that poor nutrition plays an important role in the decline in muscle strength and physical function in older persons and, consequently, may contribute to the disablement process.

BIOGRAPHICAL SKETCH

Benedetta Bartali was born on August 29, 1974, in Florence, Italy to Gian Carlo Bartali and Luisa Manuguerra. She is the second of two daughters, and grew up in Florence where attended the Ginori Conti Institute to obtain the degree as dietitian. Benedetta sought out opportunities to apply her clinical expertise to the research setting. From 1996 to 1998, she gained experience working for the European Prospective Investigation into Cancer and Nutrition (EPIC) study, and from 1998 to 2003 her research career shifted into high gear through her involvement in InCHIANTI, an epidemiologic study on risk factors contributing to the decline in physical function in older persons. These activities have provided her with invaluable hands-on experience with primary data collection in the context of large epidemiologic studies and have helped to inform her subsequent research. In 2004, Benedetta entered Cornell University as research assistant, and in 2005 she was enrolled as a Ph.D. student in the Field of Nutritional Sciences. During this time, she developed a strong desire to pursue a career as an independent investigator at the interface of nutritional sciences and aging. Benedetta loves to dance.

For my parents and my sister who offered me unconditional love and support throughout the course of this dissertation

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

In 2003, about 15% of Europeans and 12% of Americans were aged 65 years or older. The European and American States with the highest proportion of older persons were Italy (19%) and Florida (17%). The worldwide population aged ≥65 years is projected to increase by approximately 400 million from 2001 to 2030 (He W), increasing from 15.5% to 24.3% in Europe, from 12.6% to 20.3% in North America, and from 6.0% to 12.0% in Asia. Since the health care cost per capita is 3-5 times greater for persons aged 65 years and older than for younger persons (Jacobzone S), this demographical transition will result in a substantial increase in aging-related health problems and consequently health-related costs (Jacobzone S 2000).

Disability is one of the main public health concerns related to the aging population. Although the prevalence of disability declined from 1982 to 2004 (Manton KG 2006), the absolute number of disabled older persons is projected to increase as the population ages over the next two decades (Census Bureau 2000), with consequent burden for the individual who needs assistance, for caregivers who provide formal or informal help, and for increased level of health care utilization and, in turn, health-related costs (Guralnik JM 2002). Thus, the prevention of disability and the maintenance of independence in older persons represent a priority in aging research.

The reduction of muscle strength that occurs with aging, named sarcopenia, is associated with reduced physical activity (Vandervoot AA 2001) and is considered one of the risk factors for the decline in physical function and the development of disability (Volpi E, Nazemi R 2004). The decline of physical function is associated with increased risk of other adverse health outcomes, including further declines in

function (Manton, 1988), falls (Tinetti et al. 1988; Nevitt et al. 1989), recurrent hospitalization (Koyano et al., 1986; Manton, 1988), disability (Guralnik 1995) with consequent dependence on formal and informal care providers. Thus, it is evident that sarcopenia and the reduction in physical function may strongly affect quality of life of older persons and result in substantial increase in medical and long-term care costs (Miller EA 2000). Consequently, understanding the mechanisms underlying these adverse outcomes has been identified as a high priority in the aging-related research agenda (Landefeld CS 1998; Ebrahim S 1999; Jette AM 1999). In particular, the identification of treatable risk factors represents a crucial step to prevent or reduce their potentially catastrophic consequences, and may substantially contribute to reduce health-related costs and to improve well-being in older persons.

Poor nutrition is considered as a putative and potentially modifiable factor contributing to decline in muscle strength and physical function. In particular, protein intake is the major factor responsible for muscle protein anabolism in older persons (Volpi E 2003). Thus, an adequate intake of protein is necessary to tip the balance between protein synthesis and degradation in favour of protein synthesis (Bennet et al. 1989), and is considered to play a crucial role in maintaining muscle strength. In fact, increased intake of protein has been suggested as one of the potential strategies for reducing the decline of muscle strength that occurs with aging (Evans 1992, Roberts 1995, Campbell 2001). Although results on protein supplementation have been inconsistent (Fiatarone 1994, Campbell 1995, Welle 1998, Bunout 2001), some studies strongly support this hypothesis. Schurch et al. (1998) conducted a randomized, double-blind, placebo-controlled trial and demonstrated that protein supplementation administered to patients with recent hip fracture improved clinical outcomes and muscle strength. Furthermore, Scognamiglio et al. (2005) showed that

protein supplementation in older persons with reduced physical activity significantly increased muscle strength after three months of treatment.

These findings strongly suggest that protein intake plays an important role in sarcopenia. It is important to take into consideration, however, that these studies were on the effect of protein supplementation and not of dietary protein intake. Thus, the interpretation of the results may be complicated by a number of factors. In particular, the dose of protein and amino acids that likely ranges from physiological to mega doses, the characteristics of the group that has been targeted for supplementation, and the fact that older adults tend to reduce dietary intake during use of supplements (Volpi 2003) makes it difficult to conclude that protein intake derived from habitual food consumption may play an important role in sarcopenia in the general older population.

In a previous study, we found that a low dietary intake of protein was associated with low muscle strength in a population-based study of community-living older persons (Bartali 2006). The cross-sectional design of this study, however, makes it impossible to establish whether dietary protein intake has an effect on subsequent decline in muscle strength. Consequently, population-based, longitudinal studies of older persons living in the community are urgently needed to address this gap in knowledge. In fact, the current Recommended Dietary Allowance (RDA) (Food and Nutrition Board 2001) for protein was derived from studies on nitrogen balance conducted in young men (Scrimshaw NS 1972), and whether this recommendation is adequate for the maintenance of muscle strength in older persons is unknown. Furthermore, in studying the effect of protein intake on muscle strength, it is important to consider that aging is characterized by physiological changes, such as increased concentrations of pro-inflammatory markers (Johnson TE 2006), that may alter protein metabolism and utilization. In fact, a pro-inflammatory state stimulates muscle protein

turnover (Volpi E 2003) and depresses net rates of protein synthesis (McNurlan MA 2000, Volpi E, Roubenoff 1997). Thus, the effect of pro-inflammatory state that characterizes the aging process and is associated with muscle wasting and catabolism needs to be taken into consideration to understand the effect of protein intake on muscle strength in this particular segment of the population.

Another important gap in knowledge is the effect of concentrations of nutrients on decline in physical function. Most of the studies available that consider nutrition as a factor in the pathogenesis of decline in physical function use weight loss as a proxy measure of undernutrition (Andreas E 1999, Ferrucci 2004). Weigh loss especially in the older population, however, may be caused by different factors such as the presence of diseases that increases catabolism (Roubenoff 1997). Thus, weight loss may not be an appropriate indicator of undernutrition in older persons. Previous studies suggested that concentrations of nutrients may play a role in reduction in physical function through different mechanisms. Antioxidants may play a preventive role in muscle damage by reducing oxidative injury (Clarkson 2000, Fano 2001) with subsequent muscle or neuronal cell damage (Mecocci P 1999, Coyle JT 1993) and decline in physical and cognitive function (Cesari 2004, Lu T 2004). In the InCHIANTI study, we found that low dietary intake of carotenoids and vitamin E was associated with low physical function (Cesari 2004). Vitamin E deficiency may increase oxidative stress (Clarkson PM 2000) and, consequently, may cause muscle damage (Mecocci 1999) and wasting (Moylan JS 2007). A deficiency of folate may cause abnormal cell replication, particularly in the erythropoietic system, leading to megaloblastic anemia (Wickramasinghe SN 2006). This type of anemia is caused also by the B12 deficiency (Wickramasinghe SN 2006) and may result in symptoms such as fatigue, weakness, and dementia (Goodman KI 1990; Stabler SP 2004; Dharmarajan TS 2003). Iron is the key element required for the delivery of oxygen to tissue. Iron-deficient workers with

the lowest hemoglobin concentrations had the shortest time to exhaustion (Gardner 1977) and in iron-deficient anemic women, iron supplementation increased work capacity and reduced hearth rate and lactate concentration (Gardner 1975).

Despite this strong theoretical basis, there is relatively little empirical evidence linking poor nutrition to decline in physical function. Previous studies (Snowdon DA1996) and our recent findings (Cesari 2004, Bartali 2006, Semba 2006, Bartali 2006) have shown that poor nutrition is associated with reduced physical function, frailty and disability in older persons. These studies, however, have been limited by their cross-sectional design (Snodow DA 1996, Cesari 2004, Bartali 2006) or nonrepresentative samples, including, for example, only older women with some level of difficulty in physical function (Bartali 2006, Semba 2006). Thus, there is a lack of empirical evidence on the effect of undernutrition on consequent decline in physical function in older persons. This information could open a potential avenue for the development of strategies aimed at preventing the decline in physical function and disability in older persons, with an important impact on public-health costs and quality of life of older persons and their families.

Given the potential detrimental effect of undernutrition on decline in muscle strength and physical function, another important question to address is whether physiological changes that occur with aging may affect dietary intake. Undernutrition has been identified as one of the "10 hot topics" in aging research (Morley 2004) and the term "anorexia of aging" has been coined to describe the physiologic decline in food intake that occurs with aging (Morley 2001, Morley 1997), predisposing to the development of undernutrition (Morley 1997). A large proportion of older adults living in the community have a deficient intake of nutrients according to the Recommended Dietary Allowance (Bartali 2003, Lee JS 2001). Although the pathogenesis of anorexia of aging is not well understood, it is clear that multiple

factors characterizing the aging process may contribute to its exacerbation, including psychological (e.g. cognitive function and depression), behavioral (e.g. physical activity and smoking), socio-economic (e.g. social network and economic status), pathologic (e.g. chronic diseases, use of medications, chewing problems) (Rolls BJ 1992; Casper RC 1995; Elsner RJ 2002; Donini LM 2003) factors.

Changes with aging in physiological factors, such as concentrations of specific hormones and markers of inflammation, are considered putative factors contributing to anorexia of aging (Morley 1997; Donini 2003). Ghrelin was recently identified by Kojima et al. (Kojima 1999) as an orexigenic hormone secreted primarily by the stomach and duodenum. It stimulates growth hormone secretion and increases food intake in rodents and humans by influencing mealtime-related appetite and hunger, and it has been suggested that defective ghrelin signaling contributes to energy unbalance (Inui 2004). Leptin inhibits food intake and stimulates energy expenditure by altering the transcription of specific hypothalamic neuropeptides such as the orexigenic peptides NPY (Kotz C 1998). Aging is associated with increased concentrations of inflammatory markers (Johnson TE 2006) that have been suggested to affect the sense of appetite in older persons (Morley 2007; Donini 2003). Supporting this hypothesis, in a randomized controlled trial conducted in cachectic geriatric patients, Yeh et al. (2000) found that the administration of megestrol acetate improved appetite, probably through a downregulation of cytokine synthesis and release. The effect of specific hormones and markers of inflammation on dietary intake in older persons, however, is unclear.

In conclusion, the aim of this dissertation was to test the following hypotheses: 1) a low intake of protein is associated with subsequent reduction in muscle strength (sarcopenia) and the effect of protein intake on muscle strength depends on concentrations of markers of inflammation; 2) a low concentration of nutrients is

associated with subsequent decline in physical function; 3) concentrations of ghrelin, leptin and inflammatory markers are associated with reduced dietary intake.

To test these hypotheses, I will use the InCHIANTI Study, a population-based, NIH-funded epidemiological study designed to identify the risk factors contributing to decline in physical function in older persons. This study involves over 1100 men and women aged 65 years or older living in Tuscany, Italy. Given its strong focus on muscle strength and physical function, and the comprehensive assessment of dietary intake and levels of nutrients, hormones and inflammatory markers, InCHIANTI is particularly well suited to study the questions posed in this dissertation. InCHIANTI is an Italian population-based sample, raising potential concerns about the generalizability of the findings. It is unlikely, however, that the basic biological mechanisms underlying decline in muscle strength and physical function and in dietary intake with aging differ substantially from one country to another. Furthermore, the percentage of participants in this study who used nutritional supplements was very low (4%), in contrast to the United States (Murphy SP 2007). Thus, this study provides the opportunity to evaluate the "pure effect" of poor nutrition on the decline in physical function.

To examine the effect of two hormones (ghrelin and leptin) on energy intake was one of the aims of this dissertation. Future studies, however, are needed to understand the mechanisms by which ghrelin, leptin and inflammatory markers may affect dietary intake. Furthermore, other circulating factors may play a crucial role in regulating dietary intake. In particular, the hypothalamus produces both orexigenic (such as neuropeptide Y) and anorectic peptides (such as alpha-melanocyte-stimulating hormone). These peptides, however, were not measured in InCHIANTI. Thus, further studies are needed to understand the physiological changes that occur with advanced age, as well as the relationship and interaction among different

hormones regulating dietary intake.

Furthermore, vitamin E was the only antioxidant I could use in this study. Thus, future studies including different antioxidants may help to understand whether other antioxidants would yield to the same results. It has been suggested that the antioxidant capacity of different compounds depends on several factors including the antioxidant-radical reactivity and interaction among antioxidants (Tsuchihashi, H 1995). In fact, specific non-enzymatic antioxidants such as vitamins E, C and carotenoids may interact during their recycling mechanisms (Packer JE 1979; Freisleben HJ 1993; Niki E 1995), and the resulting synergistic effect has a better antioxidant capacity than the sum of the effect of each antioxidant (Niki E 1997). Consequently, future studies are needed to verify whether a synergistic effect exists among different antioxidants/nutrients on the decline of physical function. In addition, whether the effect of low levels of nutrients in decline in physical function is mediated by decline in muscle strength needs to be evaluated. Furthermore, future studies are needed to understand the mechanisms by which low intake/level of nutrients may lead to reduction in muscle strength/physical function. In particular, oxidative stress that results from an excessive production of reactive oxygen species (ROS) may play a central role in this pathway. Figure 1 shows the general conceptual model of my dissertation.

The results of this study will address important and emerging gaps in our knowledge of the role of undernutrition on sarcopenia and physical function and in the identification of physiological changes that occur with aging that may contribute to undernutrition in this particular segment of the population. In turn, this study will help the development of screening and intervention strategies aimed at preventing undernutrition and the decline in muscle strength and in physical function, and subsequently disability, in older persons. Factors such as nutrition are particularly

desirable to study: they are generally modifiable and amenable to intervention, and are highly relevant to the general population of older persons.

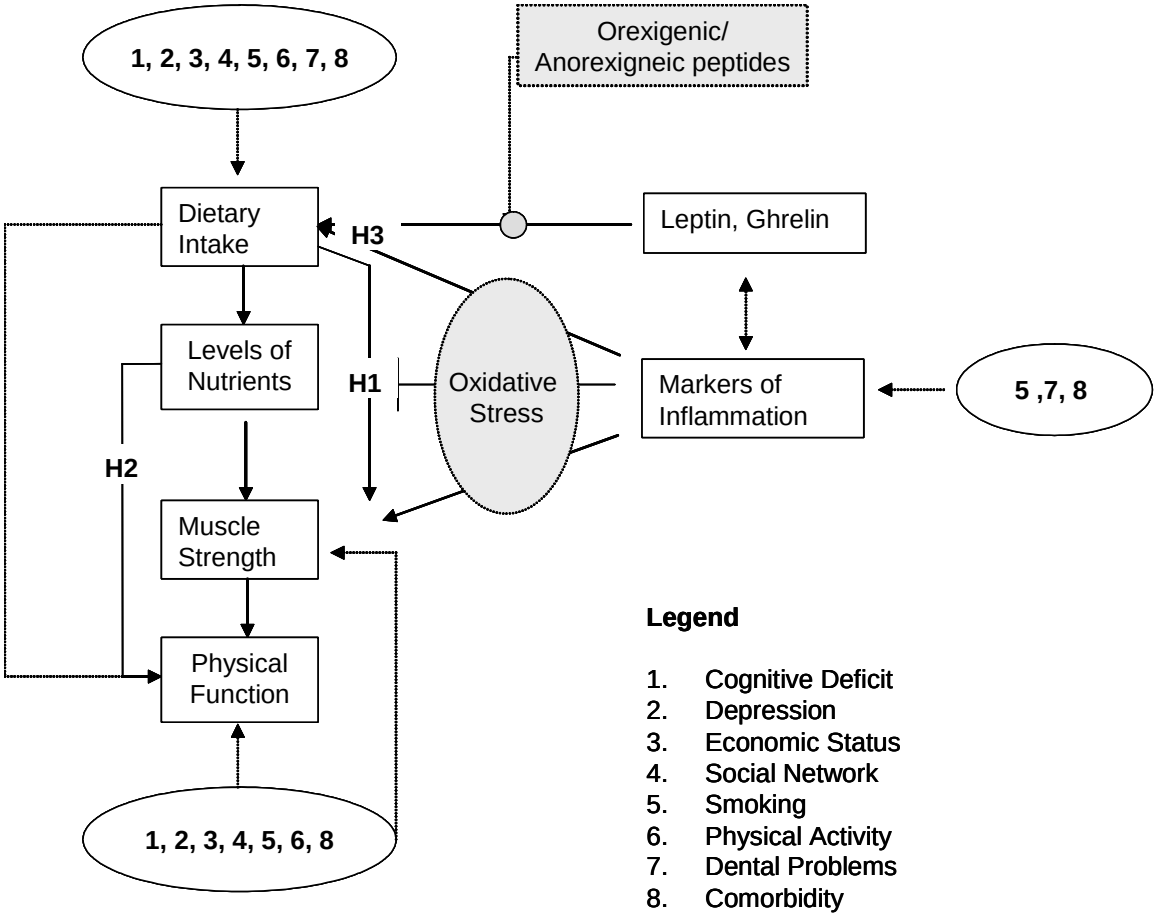

Figure 1. Conceptual framework for the hypotheses of this dissertation

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CHAPTER 2

Effect of Protein Intake on Change in Muscle Strength in Older Persons: Does Inflammation Matter?

The decline of muscle mass and strength that occurs with advanced age, termed sarcopenia (Evans WJ 2001), is a major risk factor for the development of frailty and disability, and predicts hospitalization and mortality in older persons [Fried LP 2001; Ferrucci L 2004; Rantanen T 1999; Penninx BW 2000; Rantanen T 2003). The etiology of sarcopenia is unclear, but poor nutrition and responsiveness to nutrients are potential factors contributing to its development and progression (Volpi E 2004).

In previous studies, we found that low protein intake was associated with frailty (Bartali B 2006), and that a low concentration of specific micronutrients was a predictor of disability (Bartali B 2006) in older persons living in the community. Although protein intake is primarily responsible for protein muscle anabolism in the older population (Volpi E 2003), its effect on muscle strength is unclear. The current Recommended Dietary Allowance (RDA) (Food and Nutrition Board 2001) for protein was derived from studies on nitrogen balance conducted in young men (Scrimshaw NS 1972), and whether this recommendation is adequate for the maintenance of physical function in older persons is unknown.

This study was aimed at addressing this gap in knowledge by providing empirical evidence on the effect of protein intake on muscle strength, using a longitudinal study of older persons living in the community. Since high concentrations of markers of inflammation are associated with increased muscle wasting and protein catabolism (Volpi E 2003), we also examined whether the effect of protein intake on muscle strength depends on the levels of inflammatory markers.

METHODS

InCHIANTI (Invecchiare in Chianti, aging in the Chianti area) is a study of risk factors contributing to the decline of mobility in late life, conducted in two municipalities adjacent to the city of Florence (Italy). InCHIANTI has been described in detail elsewhere (Ferrucci L 2000). In brief, 1299 participants aged 65 years or more were randomly selected from the population registry. Of the 1260 participants who were eligible (39 had died or moved away from the area), 1155 (91.6%) participated in the study. After excluding those with disability at baseline (n=116), those with missing information on knee muscle strength at baseline (n=158) or at follow-up (156), and those who refused (n=71), emigrated (n=10), or died (n=46) during the three-year follow-up, the final analytical sample included 598 persons.

Trained interviewers administered two structured questionnaires at the participant's home: 1) a questionnaire to collect information on education, socio-economical status, household composition, physical activity, functional and health status; and 2) a detailed food frequency questionnaire to collect data on dietary intake. Medical and physical assessments were performed in the study clinic by trained geriatricians and therapists, respectively.

The InCHIANTI study protocol was approved by the Italian National Institute of Research on Aging ethical committee. All subjects received an extensive description of the study procedures and all gave written informed consent.

Assessment of Dietary Intake

Data on dietary intake were collected using the food-frequency questionnaire developed for the European Prospective Investigation into Cancer and nutrition (EPIC) study (Pisani P 1997, Palli D 2000). Although the EPIC questionnaire was

originally developed for and validated in middle-aged persons, our previous study suggested that this tool provides valid estimates of dietary intake when administered to older persons (Bartali B 2004). Specific software created for EPIC was used to transform data on food consumption into daily intake of energy, macro- and micro-nutrients. A detailed description of this food-frequency questionnaire has been published elsewhere (Bartali B 2004).

Assessment of Muscle Strength

Knee extension was assessed using a hand-held dynamometer (Nicholas Manual Muscle Tester, BK-5474, Fred Sammons Inc, Burr Ridge, IL); this served in the current study as indicator of muscle strength. Participants, lying in lateral decubitus (opposite to the examined limb) with the hip and knee in 45° and 60° flexed positions, respectively, were asked to perform the task twice. The average of the results was used for the present analyses.

Measure of Markers of Inflammation

Blood samples were obtained from participants after a 12-hour fast and 15-minute rest. Aliquots of serum were stored at -80°C and not thawed until analyzed. We used ultra sensitive C-reactive protein (CRP) as indicator of inflammation. CRP was measured by ELISA using purified protein and polyclonal anti-CRP antibodies (Calbiochem, San Diego, CA) with standardization according to the WHO 1st International Reference Standard. Interleukin 6 (IL-6) and Tumor Necrosis Factor-a (TNF-α) were assessed by enzyme linked immunosorbent assay (ELISA) using ultrasensitive commercial kits (Human Ultrasensitive, BIOSOURCE International Inc., Camarillo California USA). The detectable limits were: 0.10 pg/ml for IL-6 and 0.09 pg/ml for TNF-α. The inter-assay coefficient of variation was 7% for both IL-6 and TNF-α.

Other Variables

Presence of major chronic conditions was ascertained by trained geriatricians according to standard algorithms based on information on medical history, drug treatments, signs and symptoms, medical documents, and hospital discharge records (Guralnik JM 1995). Diagnostic algorithms were modified versions of those created for the Women's Health and Aging Study (Guralnik JM 1995). Chronic conditions considered for the present analysis were: hypertension, diabetes, peripheral artery disease (PAD), stroke, angina pectoris, congestive heart failure (CHF), myocardial infarction, chronic obstructive pulmonary disease (COPD), Parkinson, cancer, and arthritis. The number of chronic conditions was used in the present analyses as continuous variable.

Weight and height were measured in standardized positions and body mass index (BMI) was calculated as kg/m2. Smoking habits were classified as never smoked, former smoker, and current smoker. Physical activity was defined as a) sedentary: completely inactive or light physical activity (i.e. walking) for less than 1 hour/week; b) light: light physical activity for 2-4 hours/week; c) moderate: light physical activity for more than 4 hours/ week or moderate physical activity (i.e., gymnastics, swimming etc.) 1-2 hours/week; d) Intense: moderate physical activity for 3 or more hours/week or walk 5 or more km/day.

Statistical Analysis

General linear models were used to evaluate the effect of protein intake at baseline on change in muscle strength over three years of follow-up. We used muscle strength at follow-up as dependent variable, and protein intake at baseline as main independent variable. Muscle strength at baseline was entered in the model as independent variable to allow prediction of the effect of protein intake at baseline on subsequent change in

muscle strength (Frongillo and Rowe). The model was adjusted for age, sex, BMI, physical activity, energy intake and presence of chronic conditions. Since protein intake provides about 15% of the total energy intake, the energy provided by protein was excluded from the total energy intake to create a variable for non-protein energy intake that was used for the adjustment.

Adjusted general linear models were used to examine the interaction between protein intake and CRP, IL-6 and TNF-α at baseline on muscle strength at follow-up. During the exploratory analyses, we used a 3-way interaction in the general linear model to exclude the possibility that the effect of the interaction between protein intake and inflammatory markers on change in muscle strength depended on the initial muscle strength. Furthermore, a 3-way interaction (protein intake*markers of inflammation*sex) was examined to verify whether the analyses had to be stratified by sex. In addition, we verified whether the effect of the interaction between protein intake and markers of inflammation on muscle strength at follow-up depended on the presence of diseases (protein intake*markers of inflammation*chronic conditions) (Table 2). To graphically show the interaction between protein intake and markers of inflammation on muscle strength and facilitate the interpretation of the results, we estimated muscle strength at follow-up according to specific values of protein intake and CRP at baseline (Figure). Additionally, the interaction between protein intake and markers of inflammation was examined selectively in persons without diseases.

Although protein intake was entered in the model as g/day, the graph was constructed to show both the values of protein intake as entered in the model (g/day) and the corresponding amount of protein intake expressed in g/kg/day to facilitate the comparison with the RDA. According to the criteria used by the Institute of Medicine to extrapolate recommendations for protein intake, the calculation of protein intake expressed in g/kg/day was based on the average weight of the study population (70

kg). Since concentrations of CRP, IL-6 and TNF-α were not normally distributed, we log-transformed these variables to perform the analyses but showed the corresponding natural values of CRP in the graph. All analyses were performed using the SAS statistical software, version 8.1 (SAS Institute Inc. 2004).

RESULTS

The comparison of characteristics between participants included and not included in the study is reported in Table 1. Participants excluded from the study were older, mostly female, with lower economical status, more sedentary, with more chronic conditions, higher levels of CRP and TNF-α and with lower energy and vegetable protein intake. The mean age of participants included in the study was 73 years and 53% were women. Descriptive information about the key variables is presented in the Appendix table. After adjustment for age, sex, BMI, physical activity, energy intake, chronic conditions, and muscle strength at baseline (Table 2), protein intake was not associated overall with change in muscle strength over three years of follow-up ($p<0.686$;). We found, however, a significant interaction between protein intake and CRP, IL-6 and TNF-α on change in muscle strength (Beta coefficient=0.020, p=0.003; Beta coefficient=0.016, p=0.058; and Beta coefficient=0.016, p=0.019; respectively). For example, increasing the log of CRP of 1 unit, the slope for the effect of protein intake on muscle strength increases of 0.02. Thus, in persons with high levels of inflammatory markers, lower protein intake was associated with a greater decline in muscle strength (Figure 2). In total, 8.1% of the population had levels of CRP $\geq$ 10, of whom 1.5% had an intake of protein < 56 g/day and 1.5% had an intake of protein > 91 g/day (Figure 2). These results were not attributable to the presence of chronic

conditions (Table 2). We also repeated the analyses selectively in participants without diseases, and the result on the interaction between protein intake and CRP on muscle strength did not substantially change (n=188; Beta =0.024; p=0.028).

The 3-way interaction term with sex (protein intake*markers of inflammation*sex) was not significant, indicating that the stratification of the analyses by sex was not justified.

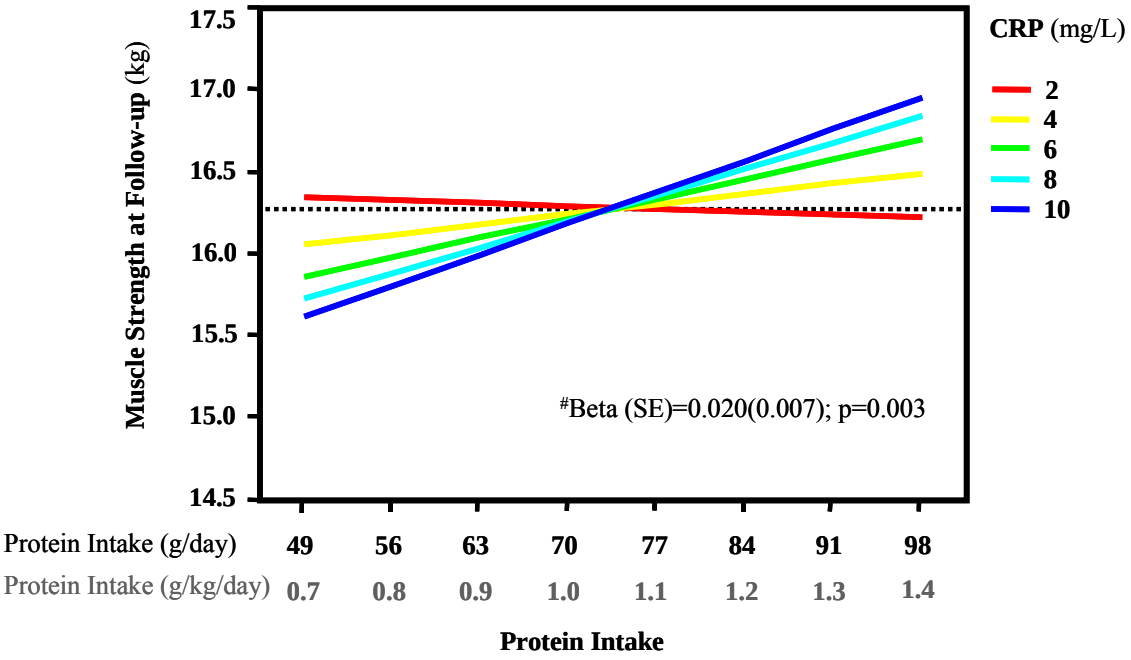

Figure 2. Predicted muscle strength* at 3-year follow-up, according to protein intake and CRP levels at baseline

* Adjusted for age, sex, BMI, energy intake, chronic conditions, physical activity, and muscle strength at baseline

^ The dashed line represents the mean muscle strength, adjusted for age and sex

The values to create this figure were derived from the model presented in Table 2 [Protein * Log(CRP)]

Table 1. Main characteristics of the study participants

General Characteristics	Included in the Study (n=598) Mean ± SD (or %)	Excluded from the Study (n=557) Mean ± SD (or %)	*p**
Age (years; range: 65.1-92.8)	72.9 ± 5.6	79.3 ± 8.2	<.0001
Gender (Female, %)	52.8	60.9	0.0060
Education (years)	5.7± 3.3	5.3 ± 3.3	0.5424
Living Alone (%)	16.9	20.7	0.1018
Economical Status (sufficient, %)	68	49	<.0001
Smoking (pack-year)	12.4 ± 20.3	12.4 ± 22.5	0.1192
Physical Activity (sedentary, %)	10.25	40.9	<.0001
Body Mass Index (kg/m^2)	27.5 $\pm$ 3.8	27.4 ± 8.2	0.1753
Number of Chronic Conditions	1.2 $\pm$ 1.1	4.6 ± 6.2	<.0001
CRP (mg/L)	3.98 $\pm$ 5.06	7.4 ± 13.2	<.0001
IL-6 ultra-sensitive (pg/ml)	1.86 $\pm$ 3.6	2.9 ± 5.07	0.1715
TNF-α (pg/ml)	3.38 $\pm$ 4.82	4.47 $\pm$ 6.43	0.0076
Energy Intake (kcal/day)	1999 ± 568	1776 ± 533	0.0026
Total Protein Intake (g/day)	77 ± 20	70.5 ± 20.5	0.0242
Vegetable Protein (g/day)	29.0 ± 9.5	25.5 ± 9.6	0.0062
Animal Protein (g/day)	48.5 ± 15.0	45.0 ± 14.7	0.1822
Muscle Strength at Baseline (kg)	15.9 ± 5.6		
Decline in Muscle Strength , %	43		

Table 2. Effect of protein intake on subsequent change in muscle strength

	Beta	**(SE)**	***p*** [#]
Protein Intake	-0.005	(0.012)	0.686
Protein*Log(CRP)	0.020	(0.007)	0.003
Protein*Log(IL-6)	0.016	(0.008)	0.058
Protein*Log(TNF-α)	0.016	(0.006)	0.019
Protein*Log(CRP)*sex	0.007	(0.014)	0.637
Protein*Log(IL-6)*sex	-0.000	(0.018)	0.986
Protein *Log(TNF-α)*sex	-0.021	(0.014)	0.145
Protein*Log(CRP)*chronic c.	-0.002	(0.006)	0.803
Protein*Log(IL-6)*chronic c.	0.003	(0.008)	0.691
Protein* Log(TNF-α)*chronic c.	-0.008	(0.006)	0.198

[#] Adjusted for age, sex, BMI, energy intake, chronic conditions, physical activity and muscle strength at

DISCUSSION

This study examined whether protein intake affects subsequent decline of muscle strength in older persons living in the community, and whether this effect was dependent on inflammation. The overall effect of protein on subsequent decline in muscle strength was not significant. We found, however, a significant interaction between protein intake and markers of inflammation on muscle strength. In persons

with high levels of inflammatory markers, lower protein intake was associated with a greater decline in muscle strength.

Previous studies found a significant association between oral aminoacids supplementation and muscle strength in older persons with low physical activity and in geriatric patients (Scordamiglio R 2005, Dehail P 2005). This is the first longitudinal study, however, on the relationship between protein intake and muscle strength.

The result on the interaction between protein intake and inflammatory markers on muscle strength could be attributable, at least in part, to the complexity of cellular and metabolic activities involved in the pro-inflammatory state that may alter the pattern of regulation of protein metabolism and may impose priorities. In fact, the systemic inflammatory response is associated with oxidative stress (Volpi E 2003) and stimulates muscle protein turnover to allow for the adaptation to this stressful condition and depresses rates of protein synthesis (Smith KL 1993). Although this process is a protein- and energy-demanding response, the amino acids released by tissue protein breakdown represent a substrate for the synthesis of numerous proteins and peptides involved in the immune system, with consequent reduction in plasma amino acid concentration (Suliman ME 2005). Previous studies have shown that amino acid availability is critical in the regulation of muscle protein metabolism (Biolo G 1997; Rennie MJ 1983). Thus, high levels of inflammatory markers in older persons may result in an increased requirement for dietary intake of proteins to create a more anabolic environment, and reduce the reliance of amino acids from muscle protein breakdown. In line with our findings, previous studies (Campbell WW 1994, Campbell WW 2001) suggested that older persons may require a higher protein intake (1.0 g/kg/day) than the current Dietary Recommended Intake (0.8 g/kg/day) (Food and Nutrition Board 2001).

The main limitation of this study is that protein intake was assessed using a food frequency questionnaire. Thus, the accuracy of the observed associations might be affected by information bias. The distribution of errors, however, is unlikely related to the outcome. Consequently, the observed results are likely to be underestimated. An important strength of this study is that we used an objective measure of muscle strength that is highly reliable (Cesari M 2004). Furthermore, these results are adjusted for non-protein energy intake and, consequently, show the "pure" effect of protein intake on muscle strength, independent of the intake of energy from other sources. We repeated, however, all the analyses adjusting for total energy intake instead of non-protein intake and the results did not substantially change.

In conclusion, we found that the overall effect of protein intake on subsequent decline in muscle strength was not significant, but there was a significant interaction between protein intake and markers of inflammation on decline in muscle strength. In persons with high levels of inflammatory markers, lower protein intake was associated with a greater decline in muscle strength, independent of the presence of diseases. These findings suggest that high levels of markers of inflammation may alter protein metabolism and the efficiency of protein utilization. Furthermore, the current recommended dietary intake of protein (0.8 g/kg/day) extrapolated from the middle-aged population may not be appropriate for the maintenance of physical function in older persons.

To our knowledge, this is the first longitudinal study on the effect of protein intake and inflammatory markers on muscle strength in older persons. Further studies are needed to confirm these results, and caution should be used in recommending higher protein intake to older persons with specific chronic diseases (e.g., kidney diseases). These results may help to understand the etiology of sarcopenia and to develop strategies aimed at preventing or delaying its debilitating consequences.

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CHAPTER 3

Low Vitamin E Concentration is a Determinant of Decline in Physical Function among Older Persons Living in the Community

The decline in physical function that occurs with aging often represents the early stage of a continuum leading to disability and other important adverse outcomes such as institutionalization. Although the prevalence of disability declined from 1982 to 2004 (Manton KG 2006), the absolute number of disabled older persons is projected to increase as the population ages over the next two decades (Census Bureau 2000), with a detrimental effect on the health-related costs and long-term care (Guralnik JM 2002). Thus, disentangling the mechanisms underlying the disablement process has been identified as a high research priority (Landefeld CS 1998; Ebrahim S 1999; Jette AM 1999) and the assessment of physical function has become an essential feature of the comprehensive clinical evaluation of older persons (Applegate WB 1990). Standardized measures such as the Short Physical Performance Battery (SPPB) (Guralnik JM 1994; Guralnik JM 1995) have been developed to study the etiology and progression of functional decline and disability.

Poor nutrition may play a role in the disabling process through different mechanisms (Bartali 2007), for example by increasing the levels of markers of inflammation and oxidative stress (Esposito K 2004, Lopez-Garcia E 2004, Lopes HF 2003, Di Mascio P 1991) with subsequent muscle or neuronal cell damage (Mecocci P 1999, Coyle JT 1993) and decline in physical and cognitive function (Cesari 2005, Lu T 2004). Despite this strong theoretical basis, there is relatively little empirical evidence linking poor nutrition to decline in physical function. Previous studies (Snodow DA 1996) and our recent findings (Cesari 2004, Bartali 2006, Semba 2006,

Bartali 2006) have shown that poor nutrition is associated with reduced physical function, frailty and disability in older persons. These studies, however, have been limited by their cross-sectional design (Snodow DA 1996, Cesari 2004, Bartali 2006) or non-representative samples, including for example only older women with some level of difficulty in physical function (Bartali 2006, Semba 2006).

The purpose of this study was to determine whether a low concentration of nutrients is associated with subsequent decline in physical function. We used data from a population-based, longitudinal study of community-living older men and women, which included objective measures of both nutritional status and physical function.

METHODS

InCHIANTI is a population-based study of risk factors contributing to decline in physical function in older persons living in two municipalities located in Tuscany, adjacent to the city of Florence. The design and methods on data collection for InCHIANTI have been described in detail elsewhere (Ferrucci 2000). In brief, persons were randomly selected from the population registry, and 1155 persons aged 65 years or older participated in the study. The response rate was 91.6%.
Data collected at baseline and during a 3-year follow-up assessment were used for the current study. As shown in Figure 3, the exclusion criteria were missing information on the Short Physical Performance Battery (SPPB) at either baseline or follow-up, and SPPB <3 at baseline (to exclude participants with very poor functional status).
The final analytical sample included 698 participants aged 65 years or older.

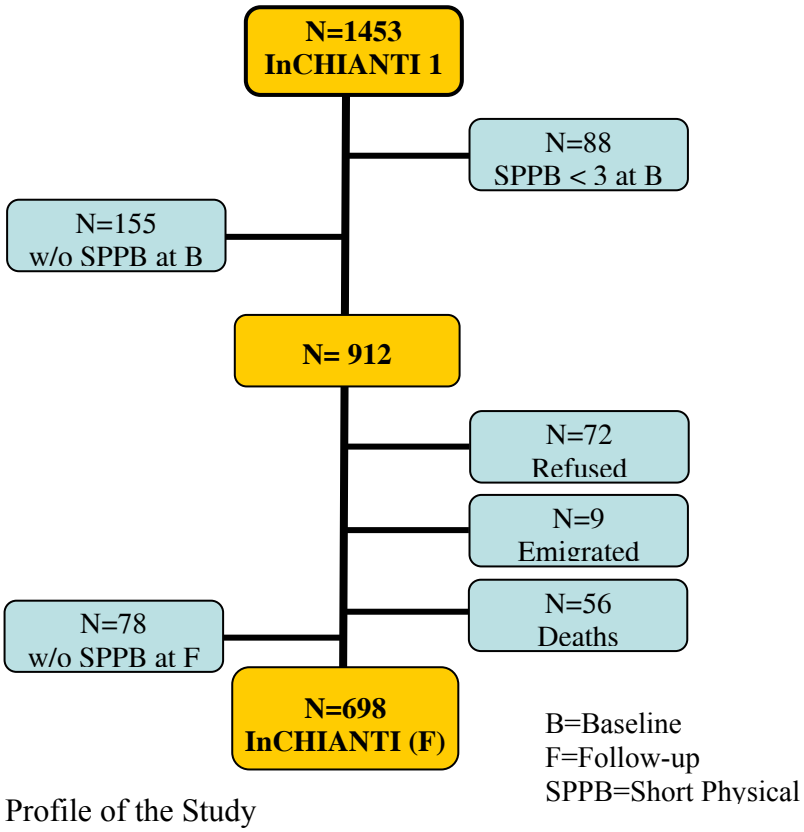

Figure 3. Profile of the Study

Trained interviewers administered a structured assessment at the participant's home, including questions on education, socio-economical status, household composition, health and functional status. Cognitive status was assessed by the Mini-Mental State Examination (MMSE) (Folstein MF 1975) and depressive symptoms were assessed using the Center for Epidemiological Studies-Depression scale (CESD) (Irwin M 1999). Participants were asked to specify their level of physical activity, which was subsequently classified as "light" if they reported walking outside their home for less than 4 hours/week (Elosua R 2005). A validated food frequency questionnaire was administered to estimate energy intake (Pisani P 1997, Bartali B 2004).

A medical history and physical assessment were performed in the study clinic by physicians and therapists, respectively. Weight and height were measured according to standard protocols (Bartali B 2002) and body mass index (BMI) was

calculated as kg/m2. The presence of major chronic conditions was established by trained geriatricians according to algorithms based on information from the medical history, drug treatments, signs and symptoms, and hospital discharge records (Guralnik, Fried 1995). The number of chronic conditions served as the indicator of comorbidity.

Assessment of Serum Nutrients

Fasting blood samples were obtained by venipuncture between 7.00 am and 10.30 am. Folate, vitamins B-6, and B-12 were obtained by centrifuging blood collected in evacuated tubes without anticoagulant and were stored at -80 °C. Vitamin B-6 was measured by HPLC (Immundiagnostik, Bensheim, Germany) and vitamin B-12 and folate by radioimmunoassay (ICN Pharmaceuticals, New York, NY). The minimum detectable concentrations were 0.6 ng/mL for folate, 0.2 ng/mL for vitamin B-6, and 75 pg/mL for vitamin B-12; the intraassay CVs were 4.1% for folate, 2.8% for vitamin B-6, and 11.2% for vitamin B-12; and the interassay CVs were 7.1% for folate, 4.1% for vitamin B-6, and 12.3% for vitamin B-12. Plasma vitamin E (α-tocopherol) concentrations were measured by reversed-phase HPLC as previously described (Martin A 1995). Triplicate analysis of the reference samples provided by the American Association for Laboratory Accreditation (Washington, DC) showed an intrabatch CV of 3% and an interbatch CV of 4.2%. 25(OH)-vitamin D was measured by RIA (DiaSorin Inc., Stillwater, MN, USA), after extraction of samples with acetonitrile. Intra- and inter-assay CVs were 8.1. Intra- and interassay CVs were <3.0 and 5.5 %, respectively. Iron was assessed using a colorimetric assay (Roche Diagnostics, Mannheim, Germany). The analytical sensitivity was 5 mg/dL, and the measuring range was 5 to 1000 mg/dL.

Assessment of Physical Function

The score on the SPPB was derived from performance on three objective tests of physical function: four-meter walking speed, repeated chair rises, and standing balance in progressively more challenging positions (Guralnik 1994). Walking speed was defined as the best performance (time) of two walks at usual pace over a four-meter course. For the chair-stand test, participants were asked to rise and sit down five times as quickly as possible with their hands folded across the chest; and performance was expressed as total time to complete the test. For the standing-balance test, participants were asked to stand in three progressively more difficult positions for 10 seconds each: side-by-side feet standing, semi-tandem, and full-tandem position.

For each of these three performance tests, participants received a score from 0 to 4, with a score of 0 indicating the inability to complete the test and 4 the highest level of performance. The scores were summed to create a total score ranging from 0 to 12 with higher scores representing better performance. Previous studies have demonstrated that older, non-disabled persons with low SPPB score are at high risk of developing disability (Guralnik 1995), and reported a test-retest correlation of 0.89 for walking speed (Nevitt MC 1989), of 0.73 for repeated chair rises (Seeman TE 1994), and of 0.97 for standing balance (Winograd CH 1994).

Statistical Analysis

Descriptive analyses were performed to provide information on general characteristics of the study population. The SPPB score at baseline was subtracted from the score at the 3-year follow-up to identify participants who declined in physical function. Because the loss of 1 point in the SPPB score is considered a clinically meaningful (Perera S 2006) and potentially modifiable change (Pahor 2006), a dichotomised variable (1=loss of at least 1 point; 0=no loss) was created and used as the primary

outcome in the current study. Exploratory analyses examined the interaction between the concentration of nutrients and SPPB score at baseline on SPPB score at follow-up to evaluate whether the effect of concentration of nutrients on change in SPPB score at follow-up differed across the levels of SPPB at baseline and allow ranking in the same category participants who lost > 1 points.

As in a previous study (Bartali 2006), low concentration for each nutrient was defined as the lowest quartile of the baseline distribution. The cut-off thresholds were 24.9 μmol/L for α-tocopherol, 275 pg/mL for vitamin B12, 4.35 ng/mL for vitamin B6, 1.9 ng/ml for folate, 30.5 μg/dL for 25(OH)-vitamin D, and 55 μg/dL for iron. This analytical approach was used because nutrient requirements for older persons are inadequately documented and cut-off for deficiencies extrapolated from the older population have not been defined (IOM 2006).

Logistic regression models were used to evaluate the association between low concentration of the specific nutrients and subsequent decline in physical function. The odds ratios were calculated for the lowest quartile of each nutrient, using the other three quartiles combined as the reference group. Four separate logistic models were used for each nutrient: 1) unadjusted; and adjusted for 2) age; 3) age and sex; 4) age, sex, education, marital status, household composition, smoking, physical activity, number of diseases, BMI, CESD, and MMSE score. Finally, the full adjusted model was further adjusted for energy intake.

Two additional and complementary analytical approaches (multiple general linear models and classification and regression trees (CART) analysis) were used to more completely evaluate the association between low concentration of nutrients and subsequent decline in physical function, and to confirm the validity of our primary results. Separate general linear models were used to examine whether serum levels of nutrients at baseline (continuous) were associated with the SPPB score at follow-up

(continuous), after adjustment for potential confounders as in the full adjusted model and the SPPB score at baseline (continuous). Finally, CART analysis was performed to identify a hierarchical order of and potentially complex interactions between the concentration of different nutrients (continuous) and the other variables included in the full logistic model (Model 4) with the outcome of decline in physical function (dichotomous). CART analysis uses recursive portioning to define the optimal cut-off point for continuous predictors and determines homogeneous groups having the largest difference in the outcome variable (minimum misclassification error rate) (Allore H 2005). Interactions between independent variables occur recursively instead of simultaneously as in linear regression. This results in a classification rule with the optima cut-point for continuous variables and is represented as a tree. Cross validation was applied and the tree with the smallest deviance (sum of squares for residuals) was considered with the optimal size (Allore H 2005). The CART analysis was performed using S-PLUS statistical software (S-PLUS 2000). All other analyses were performed using SAS version 8.1 (SAS 2000).

RESULTS

The comparison of characteristics between participants included and excluded from the study is reported in Table 3. Participants excluded from the study were older, had lower level of physical activity, and lower cognitive and physical function. Among participants included in the study, the mean age was 73.7 years, 54% were women, and the mean decline in SPPB score was 1.1 points. During the 3-year follow-up, 50.4% of the population declined of at least 1 point in SPPB score. Further descriptive information about the key variables is presented in the Appendix table.

In the unadjusted analyses (Table 4), low concentration of both vitamin E and vitamin D were significantly associated with subsequent decline in physical function, with odds ratios of 1.65 (CI=1.17-2.34, p=0.005) and 1.45 (CI=1.03-2.05, p=0.033), respectively. In the full adjusted model, the association between vitamin E and physical function remained statistically significant, with an odds ratio of 1.62 (CI=1.11-2.36; p=0.012). Thus, participants with low concentration of vitamin E were 1.62 times more likely to decline in SPPB score compared to the other group. Even after adjustment for energy intake the results did not change appreciably (OR=1.63; CI=1.12-2.38; p=0.011). As previously suggested (Traber MG 2000), we adjusted for total cholesterol the analyses on vitamin E. Since cholesterol-adjusted and cholesterol-unadjusted models yielded similar results, cholesterol-unadjusted models are presented.

Since the effect of folic acid on decline in physical function was opposite than expected (OR=0.72) and marginally significant (p=0.095), we further investigated this result and hypothesized that participants with high levels of folic acids at baseline were those who received integration of folic acid because of the presence of health-related problems. Confirming this hypothesis, we found that selectively in participants with levels of folic acid higher than the mean (3.3 ng/mL), levels of folic acid were positively and significantly associated with higher number of chronic conditions (Beta=0.41; p=0.02, after adjustment for age).

In the multivariate general linear regression analyses, only a lower concentration of vitamin E (continuous) was significantly associated with lower SPPB score at follow-up, after adjusting for potential confounders and the SPPB score at baseline (Beta=0.023; p=0.01). After further adjustment for total energy intake, the result did not substantially change (Beta=0.023; p=0.01). Finally, the effect of vitamin E on change in the SPPB score at follow-up did not depend on the initial SPPB score (interaction term: Beta =-0.005; p=0.328).

Table 3. Main characteristics of the study participants aged 65 years or older

	Included in the Study (n=698)	**Excluded from the Study** (n=457)	
	Mean ± SD (or %)	**Mean ± SD (or %)**	***p****
Age (years)	73.7 ± 6.2	79.4 ± 8.3	<.0001
Gender (Female, %)	54.4	60.3	0.051
Living Alone (%)	18.3	19.21	0.7087
Body Mass Index (kg/m^2)	27.5 ± 3.9	27.4 ± 3.4	0.242
Physical Activity			<0.0001
Sedentary	13.3	40.8	
Light	46.3	36.0	
Moderate/Intense	40.4	23.2	
Smoking (pack-year)	11.9 ± 19.6	13.1 ± 23.8	0.0196
Mini Mental State Examination	25.6+3.1	21.8+3.4	<.0001
Depression (CESD scale)	11.1+3.8	11.7+ 3.4	0.5660
Number of Diseases	0.4+ 0.7	0.6+ 0.8	0.0691
Levels of Nutrients			
Vitamin E (ng/ml)	30.6+8.3	29.2+ 7.3	0.1492
Vitamin B12 (pg/ml)	450.0+333.2	469.5+304.4	0.1631
Vitamin B6 (ng/ml)	7.2+6.2	6.2+ 9.1	0.4295
Folic Acid (ng/ml)	3.4+2.1	3.2+ 1.7	0.4634
Vitamin D (ng/ml)	53.9+37.3	43.5+ 24.7	0.0101
Iron (ng/ml)	84.8+25.2	80.0+ 23.4	0.1604
Physical Function			
SPPB at baseline	10.7+ 1.9	7.5+ 4.5	<.0001

Table 4. Logistic regression models^ examining the effect of low levels of nutrients (1st Quartile vs. upper three quartiles) on subsequent decline in physical function*

	Model 1		Model 2		Model 3		Model 4	
	OR (95%CI)	**p**	**OR (95%CI)**	**p**	**OR (95%CI)**	**p**	**OR (95%CI)**	**p**
Vit. E	1.65 (1.2-2.3)	0.005	1.54 (1.1-2.2)	0.021	1.62 (1.1-2.4)	0.010	1.62 (1.1-2.4)	0.013
Vit. B12	1.17 (0.8-1.6)	0.362	0.99 (0.7-1.4)	0.979	1.03 (0.7-1.5)	0.868	1.07 (0.7-1.5)	0.735
Vit. B6	1.33 (0.9-1.9)	0.106	1.02 (0.7-1.5)	0.905	1.06 (0.7-1.5)	0.763	1.04 (0.7-1.5)	0.824
Folic Ac.	0.81 (0.6-1.1)	0.210	0.69 (0.5-1.0)	0.045	0.71 (0.5-1.0)	0.063	0.73 (0.5-1.1)	0.095
Vit. D	1.45 (1.0-2.1)	0.033	1.08 (0.7-1.6)	0.691	0.98 (0.7-1.4)	0.908	0.93 (0.6-1.4)	0.700
Iron	1.15 (0.8-1.6)	0.418	1.11 (0.8-1.6)	0.574	1.11 (0.8-1.6)	0.584	1.12 (0.8-1.6)	0.556

^ Nutrients were entered in separate models;

*Outcome=dichotomized variable (1=loss of at least 1 point in SPPB score; 0=no loss)

Model 1: not adjusted; Model 2: adjusted for age; Model 3: adjusted for age and sex; Model 4: age, sex, education, marital status, household composition, smoking (pack-year), physical activity, number of diseases, BMI, CESD, MMSE.

As shown in Figure 4, among the 17 factors initially selected for the CART analysis (the 6 nutrients and the 11 covariates used in the fully adjusted models), age and vitamin E were identified as the strongest determinants of decline in physical function. Persons aged > 80 years had the highest risk of declining in physical function (p=84%), while those aged < 70 years had the lowest risk. Among persons aged 70 to 80 years, the strongest predictor of decline in physical function was a concentration of vitamin E < 32 umol/L (p=60%). The misclassification error rate for the CART analysis was 0.33.

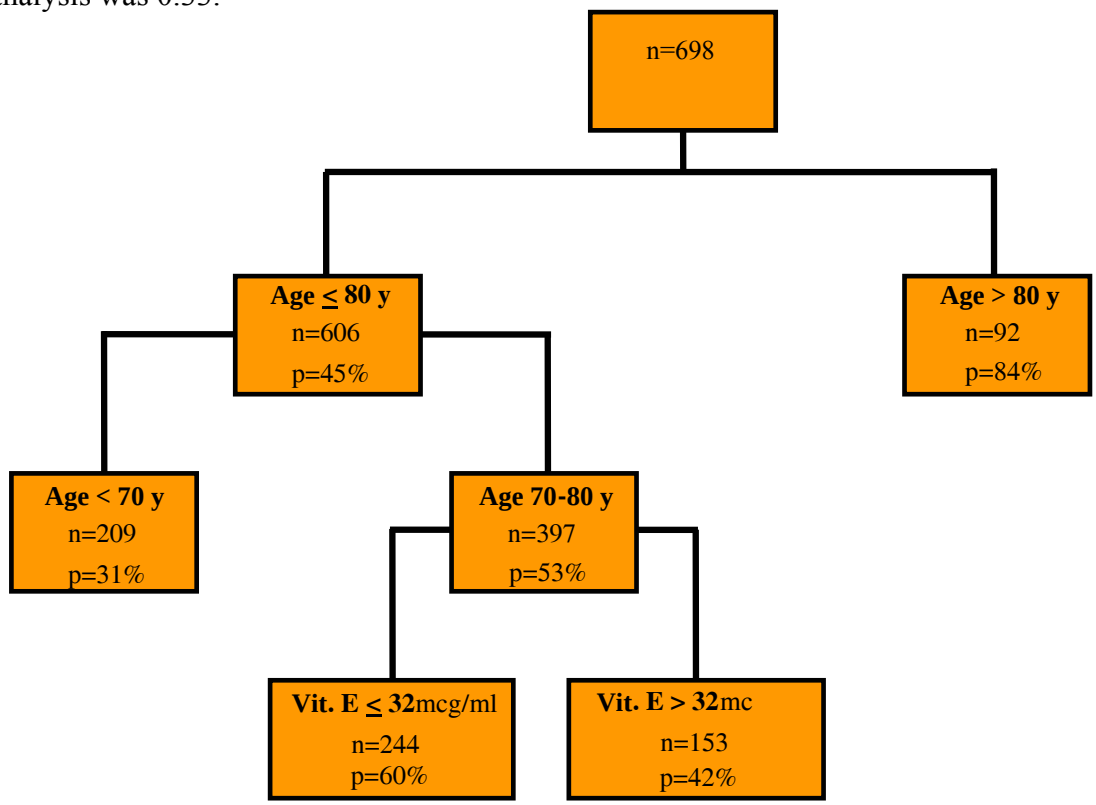

Figure 4. Classification Tree for Decline in SPPB

*Decline of at least 1 point;
Independent variables= age, sex, education, marital status, household composition, smoking physical activity, number of diseases, BMI, CESD, and MMSE
^p= probability of declining in physical function

DISCUSSION

The purpose of this study was to determine whether a low concentration of nutrients was associated with subsequent decline in physical function among older men and women living in the community. Using three different analytical approaches, we consistently found that a low concentration of vitamin E was associated with subsequent decline in physical function.

Vitamin E is the major lipid-soluble antioxidant and plays a critical role in the defence from oxidative stress by donating electrons and neutralizing free radicals. Low levels of vitamin E may affect this neutralization by creating an imbalance and, consequently, a highly reactive milieu. Since molecular oxygen promptly accepts unpaired electrons to form reactive oxygen species (ROS) (Cadenas E 2000), this imbalance may lead to excessive formation of ROS and, consequently, to oxidative stress that may cause DNA (Marnett LJ 2000), muscle (Moylan JS 2007) and neuronal (Hensley K 2006) damage, and lipid peroxidation (Voss P 2006). This chain of events may explain, at least in part, our findings on the association between low concentrations of vitamin E and subsequent decline in physical function. The hypothesis that antioxidants play a role in the etiology of decline in physical function and disability is supported by our previous findings (Bartali 2006, Semba 2006, Bartali 2006, Cesari 2004) and other studies suggesting that oxidative stress is involved in muscular fatigue (Finaud J 2006) and that antioxidants play a preventive role in muscle damage by reducing oxidative injury (Clarkson 2000, Fano 2001). Interesting, vitamin E plays a differential role in oxidative metabolism of different muscle fibers (Type I and type II). Type I fibers are plentiful in myoglobin and mitochondrial enzymes and replenish phosphocreatine more efficiently via oxidative phosphorylation than do type II fibers (Pette 1986) which theoretically lead to higher

production of free radicals. Thus, it has been suggested that type I (slow) fibers require more vitamin E than type II (fast) (Pette 1986). Furthermore, high concentration of vitamin E has been associated with increased levels of creatine kinase activity that may indicate increased skeletal muscle repair (Evans WJ 2000). In addition, vitamin E deficiency has been associated with increased lipid peroxidation (Buettner GR 1993) and risk of cardiovascular diseases (Diaz MN 1997), as well as with peripheral neuropathy and neurological deficit (Traber MG 1987; Argyriou AA 2005; Puri V 2005; Howard L 1982).

Thus, at least three different mechanisms may explain the effect of low concentration of vitamin E on subsequent decline in physical function: 1) increased oxidative stress leading to muscle, neuronal or DNA damage (Burton GW 1982), and to the exacerbation of atherosclerosis (Ryszawa N 2006) or other pathologic conditions (Diaz MN 1997, Maier CM 2002; Esposito K 2006); 2) alteration of muscle fiber types (Pette 1996; Neville 1983); and 3) peripheral neuropathy and neurological deficit (Traber MG 1987; Howard 1982). Although a low concentration of the other nutrients considered in this study could potentially play a role on the decline in physical function through different mechanisms, they were not statistically significant and this result needs to be further investigated.

To our knowledge, this is the first longitudinal study to have examined the effect of low concentrations of different nutrients on subsequent decline in physical function using a population-based sample of older men and women living in the community. We used objective measures for the evaluation of both the exposure (concentration of nutrients) and the outcome (decline in physical function). Hence, our results are not biased by self-report. Furthermore, we used an indicator of physical function derived from the assessment of three different performance tests, which increases its reliability and accuracy (Stewart 1988; Guralnik 2000). Finally, the

validity of our findings is strengthened by the use of three different analytical approaches, each of which demonstrated the same result: low vitamin E concentration was associated with subsequent decline in physical function. Of note, the cut-off of vitamin E selected by the CART analysis was 32 mcg/ml, and > 30 mcg/ml is the cut-off used to define optimal status of vitamin E (Traber MG 1996). Hence, our results have face validity. The third quartile for vitamin E in the sample was 34.90 (see Appendix table), which is close to the cut-off selected by CART. This suggests that, if there is a non-linear relationship between vitamin E and decline in physical function (e.g., a threshold), it cannot be identified within the range of the sample. This was confirmed by adding a quadratic term to the continuous analysis and finding that it was not significant.

Our findings, however, should be interpreted in light of several limitations. The loss to follow-up of respondents may have biased the results. Non participants to follow-up were older, more sedentary, had lower cognitive function and SPPB score compared to participants to follow-up. In longitudinal studies of older persons, age-related problems such as the reduction of cognitive function, morbidity and mortality are inevitable causes of attrition (Lui 1989, Deeg DJH 2002), leading to loss of power and underestimation of changes over time (Deeg DJH 2002). Thus, our results are likely to be underestimated. Furthermore, InCHIANTI is an Italian population-based sample, raising potential concerns about the generalizability of the findings. It is unlikely, however, that the basic biological mechanisms underlying decline in physical function with age differ substantially from one country to another. Because the percentage of participants in this study who used nutritional supplements was very low (4%), in contrast to the United States (Murphy SP 2007), we had a unique opportunity to evaluate the “pure effect” of poor nutrition on the decline in physical function.

In conclusion, the current study provides empirical evidence that low concentration of vitamin E is associated with subsequent decline in physical function in a population based sample of older persons living in the community. These findings, however, do not suggest that vitamin E supplementation would prevent physical function decline, but rather provide solid base that poor nutrition contributes to the decline in physical function in older persons. This study has important implications for clinical trials or interventions to reduce functional decline and, consequently, the onset of disability.

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CHAPTER 4

Effect of Leptin, Ghrelin and Inflammatory Markers on Energy Intake in Older Persons: a Longitudinal Prospective

Undernutrition is highly prevalent in older persons (Pirlich M 2001) and is associated with reduced adaptive response to physiologic and pathologic conditions (Mowé 1994), and with increased vulnerability to adverse outcomes such as morbidity (Mowé 1994), frailty (Bartali 2006) and disability (Bartali 2006). Undernutrition has been identified as one of the "10 hot topics" in aging research (Morley 2004) and the term "anorexia of aging" has been coined to describe the physiologic decline in food intake that occurs with aging (Morley 2001, Morley 1997), predisposing to the development of undernutrition (Morley 1997).

Food intake is regulated by a complex system of neural and chemical signals that modulate short and long-term feeding behaviors (Horwitz BA). Specific hormones and inflammatory markers may play a central role in orchestrating the "feeding network". In particular, ghrelin has been recently identified as a gastric peptide hormone that stimulates food intake (Kojima M 1999). Leptin is a hormone secreted by adipocytes that induces satiety, and leptin receptors are expressed by brain neurons involved in regulating energy intake (Schwartz MW). In addition, increased levels of inflammatory markers are considered important factors contributing to anorexia of aging (Morley 1997, Langhans W 1999).

Most of the studies on the effect of these hormones and inflammatory markers on energy intake, however, have been preclinical (Mattison JA 2005; Iossa S 1999; Horwitz BA 2002), have evaluated postprandial responses (Di Francesco 2006; Sturm K 2003;), and have been limited to participants with specific pathologic conditions

(Bossola M 2005; Schwenk A 2003). The long-term effect of these hormones and inflammatory markers on energy intake in the general older population is unknown. The aim of this study was to determine whether the concentration of ghrelin, leptin or inflammatory markers is associated with accelerated decline in energy intake in older persons living in the community.

METHODS

InCHIANTI (Invecchiare in Chianti, aging in the Chianti area) is a study of risk factors contributing to the decline of mobility in late life, conducted in two municipalities adjacent to the city of Florence (Italy). Details of the study have been described elsewhere (Ferrucci L 2000). In brief, 1299 participants aged 65 years or more were randomly selected from the population registry. Of the 1260 participants who were eligible (39 had died or moved away from the area), 1155 (91.6%) participated in the study. After excluding those who had cancer at baseline (n=67) and those who refused (n=101), emigrated (n=16) or died (n=127) during the three-year follow-up, the final analytical sample included 844 participants.

Trained interviewers administered two structured questionnaires at the participant's home: 1) a general questionnaire to collect information on socio-demographic characteristics, economical status, household composition, physical activity, functional and health status; and 2) a detailed food frequency questionnaire to estimate dietary intake. The InCHIANTI study protocol was approved by the ethical committee of the Italian National Institute of Research on Aging. All subjects received an extensive description of the study procedures and signed the informed consent.

Assessment of Dietary Intake

Data on dietary intake were collected using the food-frequency questionnaire developed for the European Prospective Investigation into Cancer and nutrition (EPIC) study (Pisani P 1997). Although the EPIC questionnaire was originally developed for and validated in middle-aged persons, our previous study suggested that this tool provides valid estimates of dietary intake when administered to older persons (Bartali 2004). Specific software created for EPIC was used to transform data on food consumption into daily intake of energy, macro- and micro-nutrients. A detailed description of this food-frequency questionnaire has been published elsewhere (Bartali 2004).

Assessment of Hormones and Inflammatory Markers

Blood samples were obtained from participants after a 12-hour fast and 15-minute rest. Aliquots of serum were stored at -80°C and not thawed until analyzed. Serum leptin was determined in duplicate using a commercially available enzyme linked immunosorbent assay kit (Human Endocrine LINCOplex Kit, LINCO Research, Inc., St. Charles, Missouri). The intra- and interassay coefficients of variation (CV) ranged from 2.6% to 6.2%. Ghrelin concentration was measured using ELISA assay (Human Ghrelin ELISA kit, Phoenix Pharmaceuticals, Inc., Belmont, CA, USA). The minimum detectable concentration was 0.1 ng/mL. The intra-assay CV was less then 5% and inter-assay CV was less then 14%. C-reactive protein (CRP), tumour necrosis factor (TNF)-α and interleukin-6 were used as inflammatory markers. High-sensitivity CRP was measured in duplicate by enzyme-linked immunosorbent assay using purified protein and polyclonal CRP antibodies (Calbiochem, San Diego, California) with standardization according to the World Health Organization's reference standard. The minimum detectable concentration was 0.03 mg/L, and the interassay coefficient

of variation was 5%. TNF-α and IL-6 and were assessed by enzyme linked immunosorbent assay (ELISA) using ultrasensitive commercial kits (Human Ultrasensitive, BIOSOURCE International Inc., Camarillo California USA). The detectable limits were: 0.09 pg/ml for TNF-α, and 0.10 pg/ml for IL-6. The inter-assay coefficient of variation was 7% for both TNF-α and IL-6.

Other Variables

Presence of major chronic conditions was ascertained by trained geriatricians according to standard algorithms based on information on medical history, drug treatments, signs and symptoms, medical documents, and hospital discharge records (Guralnik JM 1995). Diagnostic algorithms were modified versions of those created for the Women's Health and Aging Study (Guralnik JM 1995). Chronic conditions considered for the present analysis were: hypertension, diabetes, peripheral artery disease (PAD), stroke, angina pectoris, congestive heart failure (CHF), myocardial infarction, chronic obstructive pulmonary disease (COPD), Parkinson, cancer, and arthritis. The number of chronic conditions was used in the present analyses as continuous variable.

Statistical Analysis

General linear models were used to evaluate the effect of leptin, ghrelin and inflammatory markers on accelerated decline in energy intake during three years of follow-up. Energy intake at follow-up was used as dependent variable, and leptin, ghrelin and inflammatory markers were included in separate models as main independent variables. The analyses were adjusted for age, sex, presence of chronic conditions, and for energy intake at baseline to allow prediction of the effect of leptin, ghrelin, and inflammatory markers at baseline on accelerated change in energy intake

during three years of follow-up (Frongillo and Rowe 1999). Furthermore, general linear models were used to examine the interaction between baseline concentration of leptin, ghrelin, CRP, TNF-α, IL-6 and: 1) age; 2) sex ; 3) sex and age on energy intake at follow-up. Since it has been suggested that there may be a bidirectional relationship between hormones and cytokines (Langhans W 1999), separate general linear models were used to mutually adjust the significant associations of hormones (leptin) and inflammatory markers (CRP and IL-6) at baseline with energy intake at follow-up, taking advantage of the chronological aspect of the longitudinal data. Since Leptin, CRP, TNF-α, and IL-6 were not normally distributed, we log-transformed these variables. All analyses were performed using the SAS statistical software, version 8.1 (23).

RESULTS

The difference in general characteristics between participants included and excluded from the study is reported in Table 5. In general, women who were excluded from the sample were older ($p<0.0001$) and with lower physical activity ($p<0.0001$), reduced cognitive function ($p<0.0001$) and higher concentrations of CRP ($p=0.0048$). Descriptive information about the key variables is presented in the Appendix table. The associations between ghrelin, leptin and inflammatory markers at baseline with energy intake at follow-up are shown in Table 6. High concentration of leptin was significantly associated with accelerated decline in energy intake. In particular, an increase in leptin of 2.7 ng/mL (corresponding to 1 natural logarithm of leptin) was associated with a decrease in energy intake of 38 Kcal. The main effect of ghrelin was not statistically significant but we found a marginally significant interaction between

ghrelin and age (Beta for ghrelin=5214; Beta for ghrelin*age=-74.0, p=0.07). Increasing age of 1 unit, the slope of ghrelin decreased of 74 kcal. Thus, the slope of ghrelin in persons aged, for example, 65 years was 404 kcal (5214 -(74*65)) per unit increase of ghrelin.

Furthermore, the main effect of CRP and IL-6 on decline in energy intake was not significant, but there was a significant interaction between these inflammatory markers and sex (Beta for CRP(log)=-122; Beta for CRP(log)*sex=70.0, p=.020 Beta for IL-6(log)=-136; Beta for IL-6(log)*sex=85, p=.028). In men, high concentration of CRP and IL-6 was associated with greater decline in energy intake compared to women, after adjustment for potential confounders including chronic diseases. In particular, the sex difference was 70 kcal and 85 kcal per unit increase of log CRP and log IL-6, respectively.

The effect of CRP(log) and IL-6 (log) on energy intake was negative in males (-52 kcal and -51 kcal per unit increase of CRP(log) and IL-6(log), respectively and positive in females (18 kcal and 34 kcal per unit increase of CRP(log) and IL-6(log), respectively). When the significant associations were mutually adjusted, the results on leptin as well as the interaction between CRP and IL-6 and sex did not substantially change (Table 7). Furthermore, the findings on significant interactions were further adjusted for smoking (pack-year) and the results did not appreciably change.

Table 5. Main characteristics of the study participants at baseline

	Included in the Study (n=844)	**Excluded from the Study** (n=311)	
	Mean ± SD (or %)	**Mean ± SD (or %)**	***p****
Age (years)	74.7± 7.0	79.3 ± 8.3	<.0001
Gender (Female, %)	60.8	55.2	0.091
Living Alone (%)	17.4	22.2	0.065
BMI (kg/m^2)	27.4 ± 4.1	27.6± 4.1	0.138
Physical Activity[24]			<.0001
Sedentary	19.19	37.3	
Light	44.08	36.33	
Moderate/Intense	36.73	26.37	
Smoking (pack-year)	12.5 ± 20.9	13.1 ± 23.8	0.0836
MMSE score[25]	25.0 ± 4.0	21.6 ± 7.2	<.0001
CESD score[26]	11.3 ± 3.8	11.7 ± 3.8	0.568
Number of Diseases	0.99 ± 0.95	1.0 ± 1.0	0.239
Energy Intake (kcal)	1935 ± 560	1774 ± 555	0.056
Leptin (ng/mL)	13.37± 17.07	14.54± 18.71	0.306
Ghrelin (ng/mL)	0.28 ± 0.05	0.28± 0.06	0.968
IL-6 (pg/mL)	2.11 ± 3.53	2.94 ± 6.17	0.213
TNF-α (pg/mL)	3.75 ± 5.61	4.15 ± 5.50	0.534
CRP (μg/mL)	4.82 ± 7.73	7.48 ± 13.8	0.005

.

Table 6. Effect of hormones and inflammatory markers on energy intake during 3-year follow-up

	Beta	(SE)	*p*[1]
Leptin (log)[2]	- 38.0	(18.4)	**0.039**
Leptin (log)*age	2.43	(2.34)	0.298
Leptin (log)*sex	19.4	(37.2)	0.602
Ghrelin (ng/mL)	-309.4	(301)	0.304
Ghrelin (ng/mL)*age	-74.0	(41.2)	0.072
Ghrelin (ng/mL)*sex	-973.1	(609)	0.106
CRP (log)	-16.2	(15.5)	0.296
CRP (log)*age	-0.17	(2.07)	0.934
CRP (log)*sex[3]	70.0	(30.1)	**0.020**
TNF-α (log)	-2.58	(2.83)	0.363
TNF-α (log)*age	-0.54	(0.44)	0.227
TNF-α (log)*sex	-7.25	(5.73)	0.206
IL-6 (log)	-8.06	(20.3)	0.691
IL-6 (log)*age	-2.15	(2.47)	0.385
IL-6 (log)*sex[4]	84.7	(38.5)	**0.028**

[1] Results reported in each row were derived from separate models adjusted for age, sex (1=male; 2=female), energy intake at baseline and chronic conditions.

[2] Intercept (SE)= 1747 kcal (225);

[3] Intercept (SE)= 1735 kcal (219); Beta CRP (log)=-122;

[4] Intercept (SE)= 1701 kcal (220); Beta IL-6 (log)=-136;

Table 7. Effect of leptin, CRP and IL-6 on energy intake (kcal) after mutual adjustment

		Beta	(SE)	*p*[1]
Leptin (log)[1]		- 38.0	(18.4)	**0.039**
Adjusted also for:	IL-6	-37.5	(18.7)	0.045
	CRP	-35.1	(19.2)	0.068
CRP (log)*sex[1]		70.0	(30.1)	**0.020**
Adjusted also for:	IL-6	71.1	(30.2)	0.019
	Leptin	76.0	(31.1)	0.015
IL-6 (log)*sex[1]		84.7	(38.5)	**0.028**
Adjusted also for:	CRP	81.1	(38.6)	0.036
	Leptin	78.6	(39.2)	0.045

[1] Adjusted for age, sex and energy intake at baseline and chronic conditions

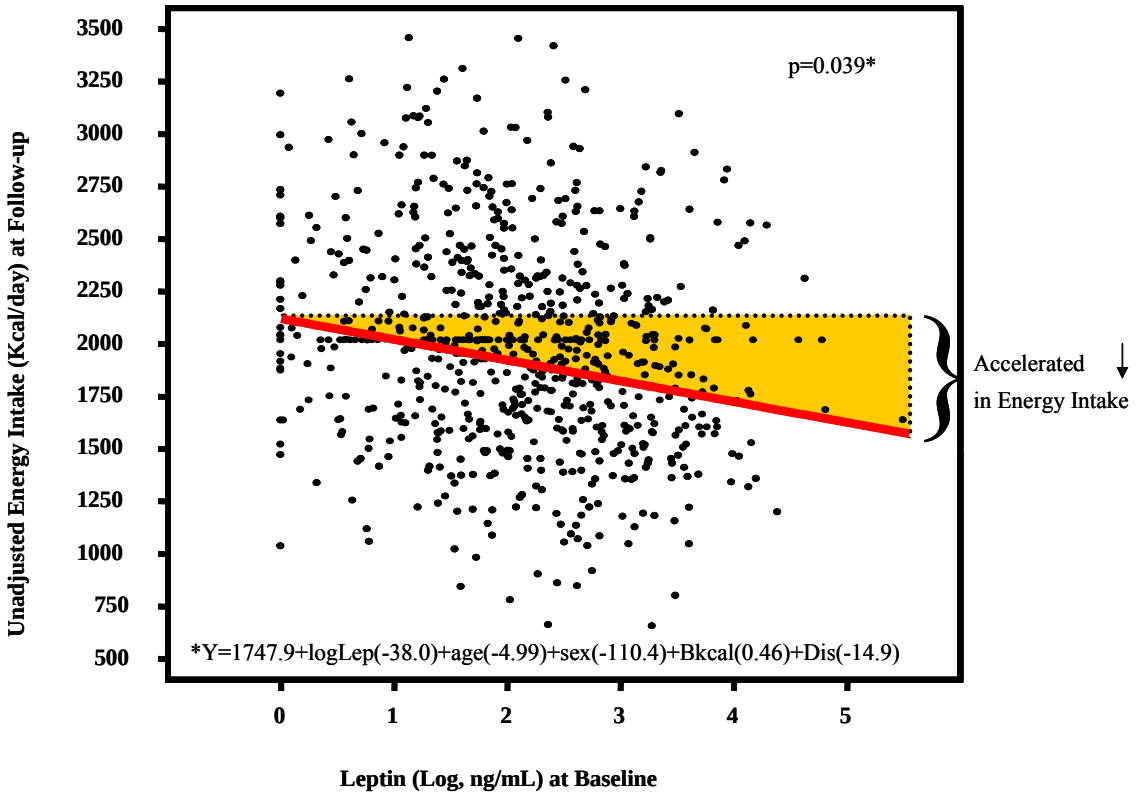

Figure 5. Relationship between Leptin (at Baseline) and Energy Intake (at Follow-up)

* After adjustment for age, sex, energy intake at baseline, number of diseases
Area in yellow indicates accelerated decline in energy intake

DISCUSSION

This study sought to examine whether concentrations of leptin, ghrelin and inflammatory markers were associated with accelerated decline in energy intake in older persons living in the community. We found that a higher concentration of leptin was significantly associated with accelerated decline in energy intake during three years of follow-up. After adjustment for CRP, this relationship was marginally significant. Furthermore, we found a significant interaction between CRP and IL-6 and

sex on change in energy intake. In men, a high concentration of both CRP and IL-6 was associated with greater decline in energy intake compared to women, independent of the presence of diseases. These findings are in line with previous studies suggesting that leptin plays a role in the long-term regulation of energy homeostasis, including energy intake (Horwitz B 2002), and that increased concentration of inflammatory markers may contribute to anorexia of aging (MacIntosh 2000). Leptin crosses the blood-brain barrier to bind receptors, and leptin receptors are expressed by brain neurons involved in regulating energy intake (Cheng CC 1997). One of the mechanisms by which leptin inhibits energy intake is by altering the transcription of hypothalamic neuropeptides, for example downregulating the orexigenic neuropeptide Y and upregulating the anorexigenic alpha-melanocyte-stimulating hormone (Jequier E 2002).

IL-6 is the major cytokine regulating the hepatic production of CRP (Heinrich PC 1990). Receptors for interleukin-6 and for leptin are present on hepatic cells and have similar action (Wang Y 1997), suggesting that leptin may stimulate the production of CRP. This hypothesis has been demonstrated in isolated human hepatocytes (Dowidar NL 2000), and accordingly we have previously found a significant association between leptin and CRP independent of IL-6 (Ble A 2006). On the other hand, it has been suggested that cytokines produce their anorectic effects by stimulating the leptin receptors (Zhang F, 1997). Thus, a complex interplay may exist between leptin and IL-6 and CRP. Our results, however, provide evidence that high concentration of leptin affects energy intake independent of the levels of CRP and IL-6. Ghrelin was not associated with consequent change in energy intake. This result is in line with previous studies suggesting that ghrelin is mainly involved in short-term regulation of food control (pre- and postprandial) while leptin is mainly involved in the long-term regulation (Di Francesco 2006; Wynne K 2005).

A limitation of the study is that we measured total ghrelin concentration whereas it has been suggested that acylated ghrelin, the active form of ghrelin, is a more accurate measure (Akamizu T 2005). Thus, further studies using acylated ghrelin are needed to confirm our findings. An important strength of this study is the nature of its design. Longitudinal research is particularly useful to study etiology (Tooth L 2005), which was the purpose of this study. Furthermore, we used a population-based sample of older persons living in the community. Consequently, our findings can be generalized to the general older population. To our knowledge, this is the first longitudinal study on the effect of leptin, ghrelin, and inflammatory markers on change in energy intake using a population-based sample of older persons living in the community.

In conclusion, we found that a high concentration of leptin was significantly associated with an accelerated decline in energy intake in older persons living in the community. Furthermore, high concentrations of both CRP and IL-6 were associated with greater decline in energy intake in men compared with women. Further studies are needed to understand the complex mechanisms underlying anorexia of aging. These results may help to understand, at list in part, the etiology of this phenomena, and ultimately inform the development of screening and intervention strategies aimed at preventing undernutrition in the older population.

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CHAPTER 5

CONCLUSION

This study sought to examine whether poor nutrition plays a role in declining in muscle strength and physical function in older persons, and whether concentrations of ghrelin, leptin and inflammatory markers are associated with reduction in dietary intake. In particular, three hypotheses were tested: 1) a low intake of protein is associated with subsequent reduction in muscle strength (sarcopenia) and the effect of protein intake on muscle strength depends on concentrations of markers of inflammation; 2) a low concentration of nutrients is associated with subsequent decline in physical function; 3) concentrations of ghrelin, leptin and inflammatory markers are associated with reduced dietary intake.

These results demonstrated that protein intake is not associated overall with increased muscle strength in older persons. We found, however, that in older persons with high levels of inflammatory markers, lower protein intake was associated with a greater decline in muscle strength, and a low concentration of vitamin E was associated with subsequent decline in physical function. Furthermore, high concentrations of leptin and inflammatory markers predicted accelerated decline in dietary intake.

This is the first population-based, longitudinal study on the effect of poor nutrition on decline in muscle strength and physical function, and on the effect of ghrelin, leptin and inflammatory markers on anorexia of aging. This pioneer study provides empirical evidence that poor nutrition plays an important role in the decline in muscle strength and physical function in older persons and, consequently, may contribute to the disablement process. Furthermore, increased concentrations of leptin

and inflammatory markers may explain, at least in part, the exacerbation of anorexia of aging and, consequently, poor nutrition in older persons.

Further studies are needed to evaluate whether and to what extent sarcopenia mediates the relationship between undernutrition and subsequent decline in physical function, and to understand the complex biological mechanisms underlying the decline in muscle strength, in physical function and anorexia of aging. These findings, however, may help to understand the etiology of sarcopenia and the reduction of physical function, and to identify the physiological factors contributing to poor nutrition in this particular segment of the population.

With the identified factors (leptin, inflammatory markers, age), it may be possible to target older persons who are at risk of undernutrition and/or nutrition-related decline in muscle strength and in physical function and, consequently, those who could benefit from nutritional intervention. In particular, older persons with high concentrations of leptin and inflammatory markers may be at risk of decline in dietary intake. Although protein intake does not have an overall effect on muscle strength in older persons, we identified the segment of the older population in which a low protein intake may have a detrimental effect on muscle strength. Selectively in persons with high levels of inflammatory markers, a low intake of protein was associated with subsequent decline in physical function. Furthermore, the study on the relationship between levels of nutrients and physical function showed that a low concentration of vitamin E is associated with subsequent decline in physical function. In particular, we identified the segment of the older population in which a low concentration of vitamin E is associated with subsequent decline in physical function. In fact, we found that participants at highest and lowest risk of decline in physical function independent were those aged $\geq$ 80 and < 70 years, respectively, and low levels of vitamin E was the strongest predictor of decline in physical function in participants aged from 70 to 80

years old. Using the cut-off of vitamin E defined by the regression tree analyses (32 mcg/ml), we found that the sensitivity for this test was 61%. This suggests that a screening test to identify older persons who are at risk of decline in physical function cannot be based only on age and levels of vitamin E. This study, however, identified the age-group (from 70 to 80 years old) in which older persons are more vulnerable to decline in physical function and may benefit from nutritional intervention. Thus, this study has important implications for the development of screening and intervention strategies aimed at preventing poor nutrition and reducing the decline in muscle strength and physical function with aging. Finally, these results may help to understand the disablement process and to prevent its detrimental consequences, contributing to reduced health-related costs and improved well-being of older persons.

APPENDIX

DISTRIBUTION OF THE MAIN VARIABLES OF INTEREST

Chapter 2							
	Mean	**SD**	**Median**	**IR**	**Range**	**Q1 (25%)**	**Q3 (75%)**
Protein (g/day)	77.5	20.5	76.3	26.9	133.7	62.5	89.5
CRP (mg/L)	3.98	5.06	2.35	3.57	47.85	1.23	4.80
IL-6 (pg/ml)	1.86	3.60	1.28	1.15	77.99	0.75	1.9
TNF-α (pg/ml)	3.38	4.82	1.90	1.52	38.8	1.44	2.96
Chapter 3							
Vit E (µmol/L)	30.2	8.35	29.13	10.50	76.89	24.40	34.90
Vit B_{12} (pg/mL)	458	345	370	225	1945	275	500
Vit B_6 (ng/mL)	7.01	6.47	5.88	4.27	91.7	4.06	8.33
Fol Ac(ng/mL)	3.24	1.93	2.80	2.10	19.70	2.0	4.1
Vit D (nmol/L)	52.0	36.8	303	39.2	303.5	28.3	67.5
Iron (µg/dL)	84.3	25.3	83	32	181	67	99
Chapter 4							
Ghrelin	0.28	0.06	0.28	0.078	0.078	0.24	0.32
Leptin	13.37	17.1	8.24	12.4	257	4.03	16.4
CRP (mg/L)	4.82	7.73	2.67	4.12	110	1.27	5.42
IL-6 (pg/mL)	2.11	3.53	1.40	1.28	77.98	0.84	2.12
TNF-α (pg/mL)	3.75	5.61	1.98	1.72	38.8	1.49	3.21