Universidad del País Vasco
Euskal Herriko Unibertsitatea

NAZIOARTEKO
BIKAINTASUN
CAMPUSA
CAMPUS DE
EXCELENCIA
INTERNACIONAL

INTERNATIONAL DOCTORAL THESIS

NUTRITIONAL STATUS AND PHYSICAL FUNCTION IN HOSPITALIZED OLDER ADULTS:

Effects of a 12-Week Resistance Training Program with Leucine-Enriched Protein Supplementation Post-Exercise Immediately After Discharge

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2022

The current International Doctoral Thesis is presented as a *compendium* of four studies. The references of these four studies included in this work are the following:

Study I: Amasene, M., Besga, A., Medrano, M. et al. Nutritional status and physical performance using handgrip and SPPB tests in hospitalized older adults. *Clinical Nutrition*. 2021;40(11): 5547-5555.

Study II: Amasene, M., Medrano, M., Echeverria, I. et al. Malnutrition and Poor Physical Function are Associated with Higher Comorbidity Index in Hospitalized Older Adults. *Frontiers in Nutrition.* 2022; 9:920485

Study III: Amasene, M., Besga, A., Echeverria, I. et al. Effects of Leucine-Enriched Whey Protein Supplementation on Physical Function in Post-Hospitalized Older Adults Participating in 12-Weeks of Resistance Training Program: A Randomized Controlled Trial. *Nutrients*. 2019;11(10):2337.

Study IV: Amasene, M., Cadenas-Sanchez, C., Echeverria, I. et al. Effects of Resistance Training Intervention along with Leucine-Enriched Whey Protein Supplementation on Sarcopenia and Frailty in Post-Hospitalized Older Adults: Preliminary Findings of a Randomized Controlled Trial. *J Clin Med*. 2022;11(1):97.

ACKNOWLEDGEMENTS/ESKERRAK/AGRADECIMIENTOS

Lehenik eta behin, mila esker nire zuzendariari, eskerrik asko Idoia urte hauetan irakatsitako guztiagatik bai maila profesionalean zein pertsonalean. Eskerrik asko bide honetan eskainitako sostenguagatik, zalantzarik gabe ezinbestekoa izan dena aurrera jarraitzeko. Eskerrik asko lehenengo momentutik nigan jarritako konfiantzagatik eta nigan sinisteagatik. Askotan hitz egin izan dugu niri suertatu zaidan doktoretza prozesu hau ez dela izan politena ez errezena. Momentu zail ugari izan ditut eta dena bertan behera uzteko zorian egon naiz hainbat momentutan. Orain atzera begira eskerrak eman nahi dizkizut momentu guzti horietan nire alboan egoteagatik, errespetuz eta munduko pazientzia guztiarekin nire alboan egon zara eta. Eskerrik asko beti nire burua azaltzeko aukera emateagatik eta behar nuen denbora eta espazioa emateagatik. Esker oneko hitzak baino ez ditut zuretako Idoia, behin eta berriz eskerrik asko. Azkenik, eskerrik asko zurekin lan egiteko aukera izan dugun guztiontzat eredu izateagatik, harro gaude eta bizi ditugun garai hauetan zu bezalako emakume bat eredu bezala alboan izatea bai maila profesionalean zein pertsonalean lujo bat da.

Aria jarraituz ikerketa taldeko kide guztiei eskerrak eman nahiko nizkieke. Eskerrik asko guztiei talde honen parte izateagatik. Gracias María, gracias por enseñarme la constancia y la pasión por lo que haces. He tenido la gran suerte de trabajar a tu lado durante estos años en los cuales he aprendido de tu profesionalidad, y estoy segura de que no hubiese llegado hasta aquí sin tu ayuda y apoyo. Eskerrik asko Maddi, zure energia ezinbestekoa da guztiontzat eta gu talde bat moduan sentitzearen arrazoi nagusienetariko bat zara. Eskerrik asko Jon, eskerrik asko Maddirekin batera burutzen duzun lan guztiagatik eta biok batera sortu duzuen talde kohesioagatik. Gracias Cristina, gracias por ese instante en el que decidiste venir a trabajar a Iruña. Es una suerte haber tenido la oportunidad de trabajar junto a ti, pero es que además de tu profesionalidad también nos has aportado toda tu pasión por el mundo de la investigación, y yo personalmente no puedo más que agradecerte por ello, ya que gracias a ti tuve la motivación y la oportunidad de realizar la estancia en Estocolmo. Thanks to my mates in Stockholm, Christina, Maria, Emmie, Nuria, but especially to Christine and Marie for giving me the opportunity to work with them and be part of their research group.

Jarraian ikerketa proiektua zein tesi hau posible izatea ahalbidetu duten guztiei eskerrak eman nahiko nizkieke. Hasteko, eskerrik asko Euskal Herriko Unibertsitateari doktoretza tesia burutzeko diru laguntza esleitzeagatik eta Eusko Jaurlaritzari ere ikerketa proiektua martxan jartzeko diru laguntza esleitzeagatik. Eskerrik asko Bittor nire tutore izateko gonbidapena onartzeagatik eta beraz tesi honen parte izateagatik. Hala ere, tesi hau ez litzake posible izango ikerketa proiektuan zehar eta ostean izan ditudan lankide guzti horiek gabe. Eskerrik asko

Ariadna, sin duda que sin tu liderazgo y apoyo en el hospital no hubiésemos podido seguir adelante con el proyecto de investigación. A pesar de las diferencias creo que hemos hecho un gran trabajo y mirando hacia atrás no puedo más que agradecerte haber sido parte de este proceso. Iñaki, eskerrik asko, eskerrik asko bide guzti honetan nire alboan egoteagatik hasieratik. Ikerketa proiektuaren atal garrantzitsuenetariko bat izan zara, zure esfortzu eta izateko era tematsu hori gabe ez ginake honaino iritsiko eta. Ane eta Miriam, nire neskak, eskerrak proiektu honen parte izatera iritsi zinetela! Nire sostengu izan zineten momentu zail eta on guztietan, zuek zarete ni prozesu honen amaieraraino iristearen arrazoi handienetariko bat. Muchas gracias Cristina, entraste como la psicóloga del proyecto y acabaste siendo la mía personal, gracias por todo tu apoyo y creer siempre en mí. Sin duda, agradecer también a todos los alumno/as que han pasado por este proyecto de investigación, Cristina, Nekane, Garbiñe, Julen, Guillermo … no hubiésemos podido sacar todo el trabajo adelante sin vosotros/as. Eskerrik asko Ana Rodriguez-Larrad eta Jon Irazusta, zalantzarik gabe tesi honen parte garrantzitsu bat izan zarete eta esker oneko hitzak besterik ez ditut eskaini didazuen arreta eta denboragatik, baita zuen profesionaltasunagandik ikasteko aukera emateagatik. Por último, agradecer tanto al hospital y su personal, como a todas las familias que han participado que son el pilar de este proyecto de investigación.

Ibilbide honi zentzua eman dioten guztiei eta momentu oro beharrezko indarra eta motibazioa mantentzen lagundu didaten pertsonei eskerrak eman nahiko nizkieke. Lehenengo, prozesu honi esker ezagutu ditudan bi pertsona berezirekin hasiko naiz. Pablete zurekin hasten naiz batera hasi ginelako ikerketa mundu honetan, eta nahiz eta bide ezberdinak jarraitu ditugun beti egon zara presente, eskerrik asko zure umore berezi horregatik eta eman dizkidazun momentu alai guztiengatik! Leiretxo, praktiketako ikasle bezala sartu zinen taldean eta orain nire eredu bilakatu zara. Eskerrik asko zurekin konpartitu ditudan une guztiengatik eta ziur nago are hobeak daudela gure zain. Orain banoa herrira, lehenengo eskaladak eman dizkidan lagunengana, momentu askotan jakin gabe haiek izan direlako indarra eta motibazioa eman didatenak, eta orain Arrate ere eskerrik asko talde berezi hontara batzeagatik, zoragarrixa zare. Herritik auzora, eskerrik asko Agirre laguntasun berezi honegatik ezingo neuke bizilagun hobeagorik izan, eskerrik asko Kepa zure bizitasunagatik eta konpartitutako barre algara guztiengatik, eskerrik asko Txolo bide lagun bilakatzeagatik eta laster bizilagun ere! Auzoa familia txiki baten bilakatzen ari da zuoi esker.

Familiaz ari garela, eskerrak eman nahiko nizkieke kuadrillari, beti alboan egoteagatik eta urte guzti hauetan eman didaten sostenguarengatik. Unibertsitateak eman zidan lagunik bereziena eta gaur egun nire familia bilakatu den Lideri ere eskerrak eman nahi dizkiot irakatsi didan guztiagatik eta nire alboan egoteagatik une oro. Mila esker Goiatz eta Eneko oparirik

politxena in doztazuelako, desietan nau etorriko dien momentu guztiak zuekin konpartitzeko eta prozesu politx honen parte izateko. Eskerrik asko Iñaki eta Anais, ezingo neuke familia hobeagorik eskatu.

Azkenik, nire bizitzako pertsona garrantzitsuenei eskaini nahiko nieke tarte hau, haiei esker hazi naizelako gaur egun naizen pertsonan bilakatu arte. Eskerrik asko ama, aita eta Andoni, guztiaren gainetik beti nire alboan egoteagatik eta zuon maitasun eta babes guztiarengatik. Agurtzane, Iñaki eta amama eskerrik asko egon zineten momentu guztiengatik, zuon falta izugarri sumatzen dut, beti egongo da zuon presentzia egiten dudan guztian. Amaitzeko, eskerrik handienak zuretzat Josu, zu gabe ez nintzateke honaino iritsiko, eskerrik asko nigan sinisteagatik eta beti aurrera egitera bultzatzeagatik, nire alderdirik hoberena ateratzeagatik mila esker Josu.

CONTENTS

ABSTRACT

The World Health Organization (WHO) estimates that by 2050 the number of persons aged 60 years and older will be doubled, being Spain one of the European countries with the highest prevalence. However, healthspan has not been rose in accordance with life expectancy. The aging process *per se* entails many biological and physiological changes that make older adults more vulnerable to the development of acute and/or chronic diseases, and thereby to hospitalization. Maintaining an optimal nutritional status and physical function, is crucial as both are considered important factors determining many of the consequences of the aging process and therefore key factors affecting quality of life. There is growing evidence that links nutrition to muscle mass, strength, and physical function in older adults. Hence, poor nutritional status might derive in malnutrition and one of the most critical consequences of it is the loss of muscle mass, whereas sarcopenia and frailty, which are characterized by muscle weakness (reduced muscle mass and strength) and thereby poor physical function, both can be preceded by malnutrition. Malnutrition, sarcopenia and frailty are conditions highly prevalent within older adults and contribute to the vulnerability seen in this population. Likewise, these 3 conditions have been associated to longer hospital stay, greater readmission rates as well as higher risk of negative clinical outcomes and mortality in older adults. However, in clinical settings the assessment of these conditions is challenging due to time and/or space limitations, and because health-care professionals must deal with other health conditions too. So, considering that malnutrition, sarcopenia and frailty share some same characteristics within their diagnostic criteria, it would be of great interest to examine the association between nutritional assessment tools and performance based physical tests and try to identify tests that might help on the screening of more than one condition.

Interventions that combine nutrition and physical exercise immediately after discharge are being proposed as the best choice to accelerate recovery and avoid hospital readmission. It is well-known that muscle contraction plays an important role in the sensitivity of old muscle to anabolic factors such as dietary protein and/or amino acid. In older adults, it has been suggested that after a resistance training program the supplementation with enriched protein would lead to greater improvements on muscle mass and strength, and physical function. Resistance training is recognized as the most potent stimulus to increase muscle mass and strength and to improve physical function. Hence, it is proposed as the first-line strategy to combat sarcopenia and frailty. In this regard, there is growing interest to know how muscle contraction-induced myokines response to resistance training, if the benefits obtained with a resistance training

program could be mediated by myokines and to better understand their role in muscle weakness related conditions, such as sarcopenia and frailty.

Our hypothesis was that a resistance training program along with leucine-enriched protein supplementation immediately after discharge will improve or even reverse the detrimental effects of hospitalization on nutritional status and physical function in older adults. Hence, those older adults with sarcopenia and/or frailty might benefit most from this nutritional and exercise intervention, and those improvements might be highlighted by changes in plasma myokine concentrations.

To test our hypothesis, we first contextualized the nutritional and physical status, and the associated comorbidity risk in older hospitalized adults at the internal medicine service of the Araba University Hospital in Vitoria-Gasteiz (Spain). For this objective, a cross-sectional study was carried out that aimed: I) to describe the nutritional and physical status and characterize the physical functional status of hospitalized older adults aged ≥ 70 years old, and II) to analyse the association between the Charlson Comorbidity Index (CCI) with nutritional status and physical function of those hospitalized older adults. Then, to test the hypothesis, a randomized controlled trial was designed aiming: III) to examine the effects of a resistance training program along with post-exercise whey protein supplementation enriched with leucine on muscle mass and strength gains in a post-hospitalized older adults' population aged ≥ 70 years old, and IV) to examine the effects of the same resistance training program with enriched protein supplementation on sarcopenia and frailty status these participants.

Four works were carried out in order to address the stablished aims. The conclusions from the current Thesis are: I) a high percentage of the hospitalized older adults at the internal medicine service of the Araba University Hospital in Vitoria-Gasteiz were at risk of malnutrition or malnourished, and showed an impaired physical function compared to their healthy counterparts. This decline within different physical tests was associated with worse nutritional status; II) handgrip strength and the Short Physical Performance Battery (SPPB), as well as its subtests, might help to complement the usual nutritional screening in hospitalized older adults. Hence, it seems that when physical function assessment is not feasible, nutritional status assessed by the Mini Nutritional Assessment-Short Form (MNA-SF) might help to predict poor physical function in this population; III) malnutrition and frailty increased the risk to be classified as at severe comorbidity according to the CCI, whereas being unfit for handgrip strength did not increase the risk. However, it seems that frailty might be a major contributor to the CCI increase than nutritional status, as older inpatients classified as non-frail had lower values of CCI regardless of their nutritional status. Nevertheless, the results of the current International

Doctoral Thesis suggest that the use of the MNA-SF and the SPPB in geriatric hospital patients might help to predict poor clinical outcomes; IV) resistance training should be considered first-line strategy to maintain muscle mass and increase gains in physical function parameters immediately after discharge in older adults. Specifically, 12 weeks of supervised resistance training with one-hour session over two days/week seems enough to enhance strength and physical function variables in this population. No additional beneficial effects are seeing with leucine-enriched protein supplementation post-exercise, but its potential cannot be discarded. Further studies are needed regarding protein supplementation in post-hospitalized older adults; V) resistance training should be considered a primary countermeasure to combat and/or prevent sarcopenia and frailty in post-hospitalized older adults. However, the additional effects of an enriched-protein supplementation with resistance training to combat these conditions needs to be further studied. Findings regarding myokines are still contradictory, and the result from the current Thesis should be taken with caution. To contrast our results, future studies are needed with larger sample sizes to understand how myostatin responds to training stimuli at the cellular level as well as at systemic level and if these responses correspond with the training outcomes observed in different contexts.

The findings of the present Thesis might have clinical implications for the management of the risk of malnutrition and/or poor physical function in hospitalized older adults. Hence, the use of either of the studied tools could be suggested, depending on the clinical setting and/or older adults' characteristics, for a first step screening. This might be highly relevant for health-care professionals who often struggle with time-, resource- and space-limitations in their daily clinical routine, and consequently older inpatients at risk of malnutrition or malnourished and/or with poor physical function are often not identified. In addition, the current Thesis might also have clinical implications in the design of resistance training intervention programs immediately after discharge to improve physical function in older adults.

RESUMEN

La organización Mundial de la Salud (OMS) estima que en 2050 se duplicará el número de personas mayores de 60 años o más, siendo España uno de los países europeos con mayor prevalencia. Sin embargo, el estado de salud no ha mejorado de acuerdo con el aumento de la esperanza de vida. El proceso de envejecimiento per se conlleva muchos cambios biológicos y fisiológicos que hacen que los adultos mayores sean más vulnerables de cara al desarrollo de enfermedades agudas y/o crónicas, y por tanto a la hospitalización. Mantener tanto un estado nutricional óptimo como una buena función física es crucial, ya que ambos se consideran factores importantes que determinan muchas de las consecuencias del envejecimiento y, por tanto, son factores clave que afectan a la calidad de vida. Cada vez hay más evidencias que relacionan la nutrición con la masa muscular, la fuerza y la función física en las personas mayores. Un mal estado nutricional puede derivar en malnutrición y una de sus consecuencias más críticas es la pérdida de masa muscular, mientras que la sarcopenia y la fragilidad, que se caracterizan por la debilidad muscular (reducción de la masa y la fuerza muscular) y, por lo tanto, una función física deficiente, pueden ser precedidas por la malnutrición. La malnutrición, la sarcopenia y la fragilidad son condiciones prevalentes en los adultos mayores y contribuyen a la vulnerabilidad observada en esta población. Las 3 condiciones se han asociado a una estancia hospitalaria más larga, a mayores tasas de reingreso y a un mayor riesgo de resultados clínicos negativos y de mortalidad en los adultos mayores. Sin embargo, la valoración de estas condiciones es complicada debido a las limitaciones de tiempo y/o espacio en los entornos clínicos, además de que los profesionales sanitarios deben tratar también muchas otras afecciones de salud. Por lo tanto, considerando que la malnutrición, la sarcopenia y la fragilidad comparten algunas características dentro de sus criterios de diagnóstico, sería de gran interés examinar la asociación entre las herramientas de evaluación nutricional y las pruebas físicas basadas en el rendimiento, y tratar de identificar así las pruebas que podrían ayudar en la detección de más de una condición.

Las intervenciones que combinan nutrición y ejercicio físico inmediatamente después de una hospitalización son consideradas la mejor opción para acelerar la recuperación y evitar el reingreso hospitalario. Se sabe que la contracción muscular desempeña un papel importante en la sensibilidad del músculo a los factores anabólicos, como las proteínas y/o los aminoácidos, en las personas mayores. Se ha propuesto que en las personas mayores la suplementación con proteínas enriquecidas inmediatamente después de una sesión de entrenamiento de fuerza puede resultar en mayores mejoras en cuanto a ganancias de masa y fuerza muscular, y la

función física. El entrenamiento de fuerza está reconocido como el estímulo más potente para aumentar la masa y fuerza muscular y mejorar la función física. Por ello, se propone como estrategia de primera línea para combatir la sarcopenia y la fragilidad. En este sentido, existe cada vez mayor interés en conocer como responden las mioquinas, inducidas por la contracción muscular, al entrenamiento de fuerza. Mas concretamente en conocer si los beneficios obtenidos con un programa de entrenamiento de fuerza podrían estar mediados por las mioquinas y en entender mejor el papel que juegan en las condiciones relacionadas con la debilidad muscular, como la sarcopenia y la fragilidad.

Nuestra hipótesis es que un programa de entrenamiento de fuerza junto con la suplementación proteica enriquecida en leucina inmediatamente después de la hospitalización mejorará o incluso revertirá los efectos adversos de la hospitalización sobre el estado nutricional y la función física de los adultos mayores. Es más, puede que los adultos mayores con sarcopenia y/o fragilidad sean los que más se beneficien de esta intervención nutricional y de ejercicio físico, y esas mejoras puede que estén mediadas por cambios en la concentración en plasma de las mioquinas.

Para probar nuestra hipótesis, primero contextualizamos el estado nutricional y físico, y el riesgo de comorbilidad asociado en las personas mayores hospitalizadas en el servicio de medicina interna del Hospital Universitario de Álava en Vitoria-Gasteiz (España). Para ello se realizó un estudio transversal con el objetivo de: I) describir el estado nutricional y físico, y caracterizar la condición física de los adultos mayores hospitalizados de 70 años o más, y II) analizar la asociación del Índice de Comorbilidad de Charlson (ICC) con el estado nutricional y la función física de los adultos mayores hospitalizados. Luego, para probar la hipótesis, se diseñó un estudio controlado aleatorizado con el objetivo de: III) examinar los efectos de un programa de entrenamiento de fuerza junto con suplementación post ejercicio de proteína de suero de leche enriquecida con leucina sobre la masa muscular y las ganancias de fuerza en una población de adultos mayores post hospitalizados de 70 años o más, y IV) examinar los efectos de ese mismo programa de entrenamiento de fuerza junto con suplementación proteica enriquecida sobre la sarcopenia y el estado de fragilidad de los participantes.

Se han realizado cuatro trabajos para abordar los objetivos establecidos. Las conclusiones de la presente tesis doctoral son: I) un alto porcentaje de los adultos mayores hospitalizados en el servicio de medicina interna del Hospital Universitario de Álava en Vitoria-Gasteiz estaban en riesgo de malnutrición o malnutridos, y mostraban una peor función física en comparación a sus homólogos sanos. El empeoramiento del rendimiento en las diferentes

pruebas físicas se asoció a un peor estado nutricional; II) el test de prensión manual y el SPPB y sus subpruebas, pueden ayudar en las personas mayores hospitalizadas a complementar las herramientas de cribado nutricional. Además, parece que cuando la valoración de la función física no es posible, el estado nutricional según el MNA-SF podría ayudar a predecir la mala función física en esta población; III) la malnutrición y la fragilidad aumentaron el riesgo de ser clasificados dentro del grupo de comorbilidad severa según el ICC, mientras que ser *unfit*, según el test de prensión manual, no aumentó el riesgo. Sin embargo, parece que la fragilidad podría contribuir en mayor medida al aumento del ICC que el estado nutricional, ya que independientemente del estado nutricional los adultos mayores clasificados como no frágiles mostraron valores más bajos de ICC. No obstante, los resultados de la presente tesis doctoral internacional sugieren que el uso del MNA-SF y del SPPB en pacientes geriátricos hospitalizados podría ayudar a predecir resultados clínicos negativos; IV) el entrenamiento de fuerza inmediatamente después de una hospitalización debería considerarse como estrategia de primera línea en las personas mayores para mantener la masa muscular y aumentar las ganancias en los parámetros relacionados con la función física. En concreto, 12 semanas de entrenamiento de fuerza supervisado con una sesión de una hora durante dos días no consecutivos a la semana parece suficiente para mejorar las variables de fuerza y función física en esta población. No se observan efectos beneficiosos adicionales con la suplementación proteica enriquecida en leucina post ejercicio, pero su potencial no se puede descartar del todo. Se requieren más estudios que contemplen la suplementación proteica en adultos mayores post hospitalizados; V) el entrenamiento de fuerza debería considerarse como primera medida para combatir y/o prevenir la sarcopenia y la fragilidad en personas mayores post hospitalizadas. Sin embargo, se requieren más estudios para examinar los efectos adicionales de la suplementación proteica enriquecida junto con el entrenamiento de fuerza para combatir estas condiciones. Los resultados con relación a las mioquinas siguen siendo contradictorios, y los resultados de la presente tesis doctoral deben considerarse con precaución. Se requieren de futuros estudios con tamaños de muestra más grandes para contrastar nuestros resultados y así entender cómo responde la miostatina al estímulo del entrenamiento tanto a nivel celular como sistémico, y si estas respuestas se corresponden con los resultados del entrenamiento observados en diferentes contextos.

Los hallazgos de la presente tesis doctoral podrían tener implicaciones clínicas para el manejo del estado nutricional y la función física de los adultos mayores hospitalizados. Por lo tanto, dependiendo del entorno clínico y/o de las características del adulto mayor, se podría sugerir el uso de cualquiera de las herramientas estudiadas para un primer cribado. Esto puede

ser de gran relevancia para los profesionales sanitarios que a menudo tienen que lidiar con limitaciones en cuanto a tiempo, espacio y recursos en su rutina clínica diaria, y en consecuencia, a menudo los pacientes mayores en riesgo de malnutrición o malnutridos y/o con una mala función física no son identificados. Además, la presente tesis doctoral también podría tener implicaciones clínicas en el diseño de programas de intervención basados en entrenamiento de fuerza inmediatamente después de la hospitalización para mejorar la función física de los adultos mayores.

LIST OF ABBREVIATIONS

CCI, Charlson Comorbidity Index

DXA, Dual energy X-ray absorptiometry

ELISA, Enzyme-Linked Immunosorbent Assays

ESPEN, European Society for Clinical Nutrition and Metabolism

EWGSOP2, European Working Group on Sarcopenia in Older People 2

ICC, Índice de Comorbilidad de Charlson

INE, Instituto Nacional de Estadística

ISAK, International Society for the Advancement of Kinanthropometry

MNA, Mini Nutritional Assessment

MNA-SF, Mini Nutritional Assessment-Short Form

OMS, Organización Mundial de la Salud

RDA, Recommended Dietary Allowance

1-RM, 1-repetition maximum

SPPB, Short Physical Performance Battery

TSHA, Toledo Study on Healthy Aging

WHO, World Health Organization

1. GENERAL INTRODUCTION

1.1 Aging and health

1.1.1 Aging demographics

The world is facing an aging of the population. According to the World Health Organization (WHO), by 2050 the number of persons aged 60 years and older will be doubled. Hence, it is expected that between 2020 and 2050 the population of people aged 80 years or older will tripled all over the world [1]. This projection of the aging process is quite similar within the Spanish population. In Spain the percentage of the population aged over 65 is around 19.6% according to the National Institute of Statistics (Instituto Nacional de Estadística, INE), and will reach a maximum of 31.4% in 2050 [2]. These predictions made by the Eurostat forecast that in 30 years four of the European regions with the oldest populations will be in Spain [3]. Due to the COVID-19 pandemic mortality was increased in 2020, especially within older adults [4]. As a result, it could be expected that those projections would be affected by the pandemic; however, the aging trend that was predicted seems to continue [4]. The COVID-19 pandemic has evidenced even more the vulnerability of older adults, highlighting the need of adapting future health care systems to the challenge emerged by the aging of the worldwide population.

Population, by broad age group, EU-27, 2019-2100
(% of total population)
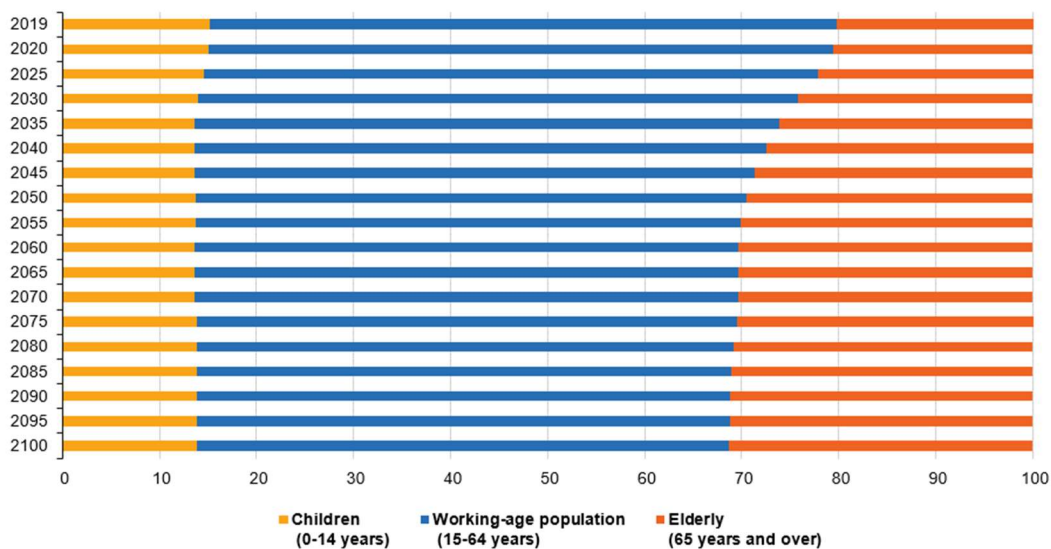
Illustration 1. The European population projections made by the Eurostat [3].

1.1.2 Age-related comorbidities

Life expectancy has been increased in the last decades, and although much of the time we live in good health, low quality of life is more common among the oldest age groups. Indeed, healthspan has not been rose in accordance with life expectancy. The prevalence of chronic

diseases has substantially increased in the last years along with the aging of the population [5,6]. According to a study carried out by Sheridan et al. [7] in 14 European countries, 50% of older adults (≥ 65-years-old) have at least 2 chronic diseases. This might be due to the fact that aging *per se* entails many biological and physiological changes that make older adults more vulnerable to the development of acute and/or chronic diseases. Different mechanisms have been described to contribute and determine the aging process [8], and many of these mechanisms are also shared by diseases [9]. Likewise, the relation between aging and diseases is much more complex. It has been proposed to consider aging and age-related diseases as part of a continuum where the appearance of diseases might accelerate the aging process and/or *vice versa* [9]. In addition, this complex interplay between aging and diseases is also determined by genetic as well as by environmental and lifestyle factors [9]. This hypothesis is supposed to explain the heterogeneity seen within older adults' population. Aging is an inevitable process, but the age-trajectory (how we get older) can be influenced by lifestyle interventional programs. Indeed, physical activity as well as nutrition are considered important factors determining many of the consequences of the aging process.

1.2 Nutritional status and physical function

Maintaining an optimal nutritional status is crucial for different aspects affecting health, and thereby quality of life [10]. Hence, as defined by the WHO an adequate nutritional status is imperative for wellbeing in higher age and is considered a modulator of healthy aging [11]. However, the nutritional status of older adults it is challenged by physiological, psychological, and social changes related to aging [12], leading to the condition termed 'anorexia of aging' (see Illustration 2 adapted from Leslie et al.[10]). All of these changes affect the appetite as well as the consumption pattern, impacting negatively on food consumption and energy and nutrients intake. Long-lasting poor dietary intake might derive in malnutrition, as deficient energy and macro- and/or micronutrient status [13]. Protein-energy malnutrition is frequent in older adults [14] and one of its most critical consequences is the loss of muscle mass [15,16]. Simple daily life activities such as, rising from a chair, walking, dressing, managing personal hygiene, shopping and so on, can be compromised by muscle weakness [17] as the latter impairs physical function.
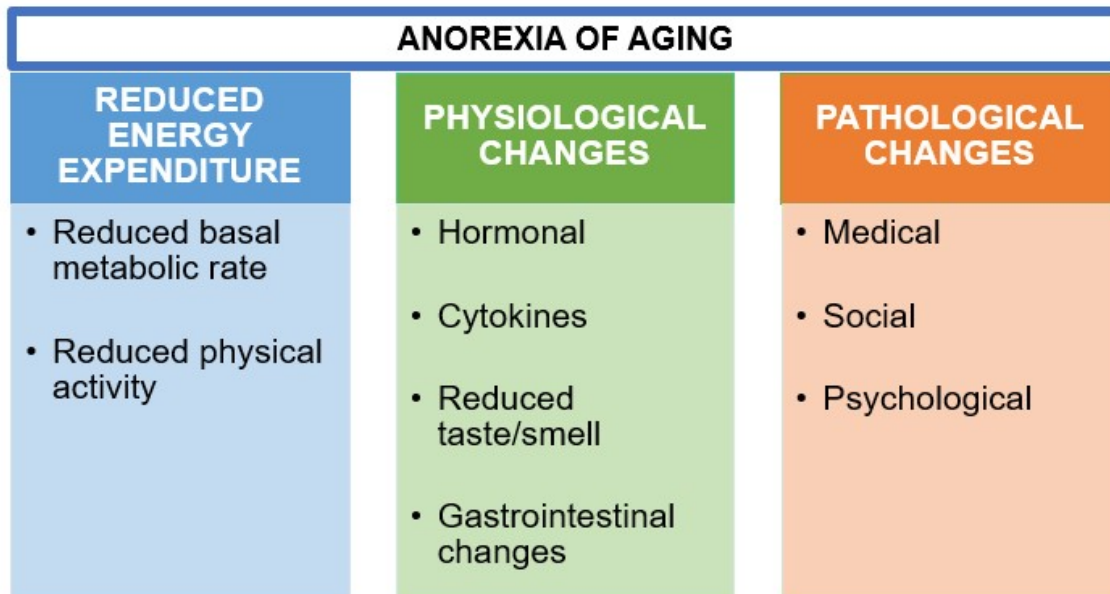
Illustration 2. Factors that affect nutritional status in older adults deriving in anorexia of aging. Adapted from Leslie et al.[10].

Thus, another modulator of healthy aging, and thereby a key factor affecting quality of life, is physical function [18]. Sarcopenia and frailty are characterized by muscle weakness due to a decline in muscle mass and strength, and thereby are also characterized by poor physical function [19,20]. Malnutrition can trigger the appearance of sarcopenia and frailty, and *vice versa*. Likewise, it is common for these conditions to partly overlap in older adults [21].

1.2.1 Risk of malnutrition and malnutrition

The aetiology and complexity of malnutrition in older adults is not well understood yet [16]. Malnutrition is a multifactorial issue and many of the determinants might be modifiable [22]. Despite, the 'anorexia of aging' mentioned before, malnutrition might also occur on a background of chronic and/or acute diseases where energy needs are increased in already vulnerable older adults [16]. Physical activity might also be a key factor, although it seems that is not a determinant of malnutrition [22]. In contrast, physical function was considered as determinant of malnutrition [22], which, in turn, is driven by physical activity levels [23–25]. Thus, not being involved in physical activity throughout the day and/or periods of inactivity, such as bed rest, might partially contribute to malnutrition as well.

The negative impacts of malnutrition or risk of malnutrition within older adults' population are well-stablished [26]. Thus, malnourished older adults are at higher risk of fracture [27], morbidity [26], and mortality [26,28], and the recovery from any disease, trauma and/or surgery intervention can be delayed [16,26]. Despite this, older adults at risk of malnutrition or

malnourished are often unrecognized and undertreated [29]. According to Leij-Halfwerk et al. [30], in Europe 23% of older adults are at high risk of malnutrition. Thereby, there is a high awareness to screen and identify those older adults at risk of malnutrition or malnourished in order to prevent and/or treat it.

1.2.2 Sarcopenia and frailty

Regardless of the nutritional status, it is well-stablished that muscle mass loss is an inevitable consequence of the aging process [31]. Janssen et al. [31] concluded that the decrease in skeletal muscle mass was around 1.9 and 1.1 kg/decade in men and women, respectively. Other authors estimated that the average rate of muscle loss among those aged 70 years and older, is around 0.5-1% per year [17]. It has been also shown that with advancing age this decline was greater in the lower body, suggesting a change in muscle distribution with aging [31]. This age-related muscle mass loss has been termed as sarcopenia, which was first defined based on muscle mass measurements [32]. Janssen et al. [33] showed that among older adults aged ≥60 years old, 45% of men and 59% of women were classified as having moderate sarcopenia, whereas 7% and 10% of men and women, respectively, were classified as having severe sarcopenia.

Currently the evidence supports that as we get older, muscle quality more than quantity might be more important [34]. Although back in 2001 Morley et al. [35] proposed that the definition of sarcopenia should also consider muscle weakness and loss of function, sarcopenia was often referred without reference to both conditions. Later, Goodpaster et al. [34], in a study of 3 years of follow-up in well-functioning older adults, demonstrated that the decline in muscle strength was 3-fold greater than that of muscle mass among both genders. Hence, it seems that muscle strength is lost at a rate of 2.5-4% per year in older adults aged around 75 years [17]. It is suggested that the functional decline seen along with the age-related muscle mass loss might be mediated by reductions in muscle strength [36]. These losses in strength might derived in functional challenges due to decreases in specific force and power [34,37]. This greater decline observed in muscle strength over muscle mass highlighted the need to not only focus on muscle mass loss but also on muscle strength as well as muscle quality when defining sarcopenia [38]. Thus, although a suitable definition of sarcopenia for clinical and research practice is still lacking, we will consider the operational definition for sarcopenia proposed by the European Working Group on Sarcopenia in Older People 2 (EWGSOP2) [38], which defines sarcopenia as 'a progressive and generalised skeletal muscle disorder'. As a result, current sarcopenia definition by the EWGSOP2 does not consider muscle mass measurement as the primary outcome. Indeed,

muscle strength measurement has come to the forefront followed by muscle quantity or quality to confirm sarcopenia and physical performance as an indicator of severity.

It has been observed that sarcopenia increases the risk for disability [39] and other adverse outcomes [40] as well as mortality [41]. Hence, sarcopenia might result in physical frailty [20]. However, frailty might also appear independently of sarcopenia due to the cumulative decline that occurs throughout life in multiple physiological systems [19]. Frailty is considered a geriatric syndrome and it is characterized by a decline in reserve and function across multiple physiological domains [42]. Thus, according to Clegg et al. [19] frailty is 'one the most problematic expression of the aging population' as it increases the risk for adverse health outcomes [43].

Frailty prevalence might vary according to the instrument used for its assessment, to the study population (e.g., community dwelling *vs.* institutionalized) and by country [44–47]. Nevertheless, it seems that all coincide on a steadily increase of frailty with age [44–47]. Frailty is a dynamic state, is not inevitable, so it can be improved, especially at its early stage (pre-frailty) [48]. Likewise, this makes of utmost importance to identify those older adults candidates to prevent and/or manage frailty.

Sarcopenia is considered a key component of frailty [20], but frailty might also accelerate the development of sarcopenia [49]. Both conditions might be preceded by the risk of malnutrition or malnutrition [50,51]. In turn, a poor nutritional status might accelerate sarcopenia and frailty by influencing on muscle mass loss and thereby, physical function [15,51,52].
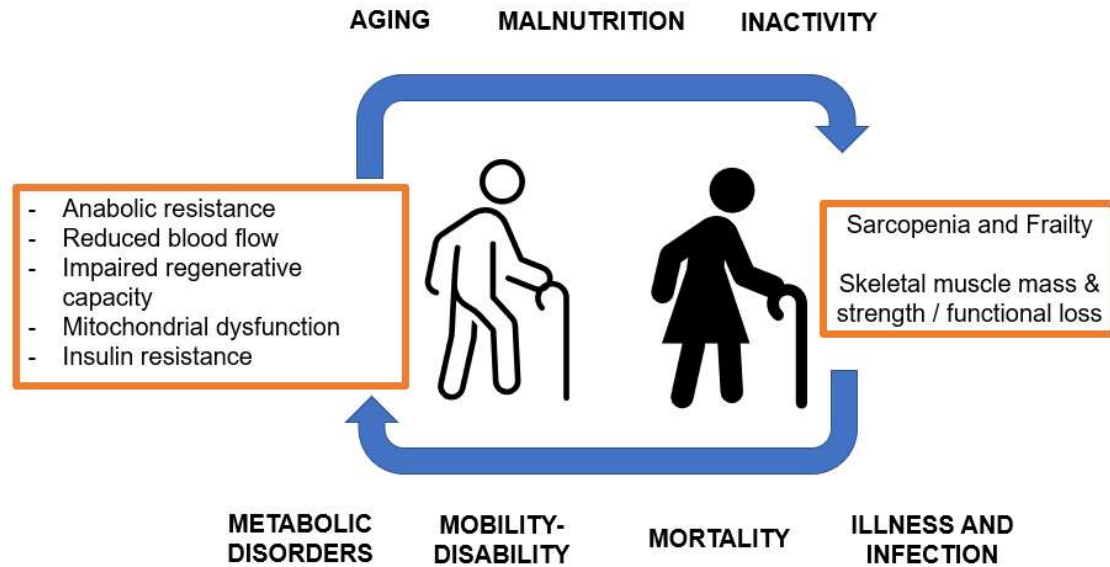
Illustration 3. Relationship between malnutrition and loss of skeletal muscle mass and function. Adapted from Landi et al. [51].

Thus, the three conditions, malnutrition, sarcopenia and frailty, coincide on muscle weakness and are often overlapped [21,38] and have adverse health outcomes [21,38,53]. As a result, older adults show higher vulnerability with increasing risk for hospitalization, longer hospital stay and time for recovery as well as mortality.

1.3 Hospitalization in older adults

1.3.1 Adverse consequences of hospitalization on nutritional status

As aforementioned, risk of malnutrition or malnutrition might occur along with a background of chronic and/or acute diseases in older adults. As a result, this already vulnerable population group is more prone to hospitalization than other younger age groups. Hence, being at risk of malnutrition or malnourished increases the risk for hospitalization [54]. The longitudinal study Toledo Study on Healthy Aging (TSHA) showed that 15% and 12.6% of community-living older adults were at risk of malnutrition and malnourished, respectively [55]. In addition, those older adults at risk of malnutrition and malnourished were more likely to have been admitted to the hospital than their well-nourished counterparts (20.16% and 30.70%, respectively, *vs* 12.44%) with the subsequent longer lengths of stays and higher annual hospitalization costs [55]. Being at risk of malnutrition or malnourished is common in older adults on admission to hospital [56–59], and this might be further aggravated during hospitalization. Hence, it has been shown in hospitalized adults (≥ 18-years-old) that within a range of 19.8-31.0% of the inpatients might suffer a decline in nutritional status during their ≥ 7 days hospital stay [60]. Different factors might contribute to these detrimental effects on

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nutritional status, but among the most important are disease-related loss of appetite and/or alter metabolism, absorption, and assimilation of nutrients [61], disease related impaired functioning of the digestive system [26], drug-related side effects [26], fasting prior to an intervention or diagnostic procedure [26], and finally poor management of patient nutrition in-hospital [26]. In addition, old age, comorbidities, and polypharmacy, which are three common characteristics within older inpatients, are considered important factors for malnutrition [59]. Indeed, malnutrition in older adults is associated with longer hospital stays and higher readmission rates [28] as it hinders the recovery from diseases, surgery, or trauma due to a worsen prognostic [26]. Thus, malnourished older adults are at a higher risk of in-hospital mortality [28] and mortality in the short- [62] and long-term [62] after discharge.

Regarding the prevalence of malnutrition risk and malnutrition among older hospitalized adults a wide range have been reported, from 6 up to 98% [29,58,59]. This wide range is mainly explained by the nutritional assessment tool used and/or according to the clinical setting, with some studies referring to acute-care, others geriatric wards or internal medicine as well as surgery wards [29,59,63,64]. Malnutrition has been referred to as the *'skeleton in the hospital closet'* as it is often overlooked, undiagnosed and untreated [63,65]. Hence, in a cross-sectional survey conducted in Europe, Schindler et al. [56] reported that nutritional screening as part of the daily routine was only implemented by half of the responding units. Screening and identifying those patients seems of great importance as being at risk of malnutrition or malnourished is common among older inpatients [65], and this carries a higher economic burden [66] mainly due to the longer length of stay in hospital and increased risk for health complications [63,66].

1.3.1.1 Tools to identify at risk inpatients in clinical settings

Some nutritional assessment tools were designed to detect malnutrition risk whereas others were designed to diagnose malnutrition [29], and many of those tools have been used interchangeably even within different settings or subgroups of patients that were originally designed for [64]. Currently, there is not a gold standard tool to screen and/or assess for malnutrition in older hospitalized adults [65]. Due to time and financial limitations, nutritional screening is preferable to nutritional assessment in clinical settings. Thus, it is recommended to first screen older inpatients for risk of malnutrition or malnutrition, and once these inpatients are identified a more comprehensive assessment should be performed by nutritional assessment tools [67].

Nutritional screening tools should be easy to use, rapid, economical, standardized, and validated as well as sensitive and specific [67]. In this regard, the Mini Nutritional Assessment (MNA) seems to be an adequate tool. It was designed specifically for older inpatients adults [68], and it has been validated in several settings [69]. The MNA showed a high inter-rated reliability and sensitivity [69]. A high sensitivity is required for screening tools [69], as it reflects de ability of the test to identify those that are at risk of malnutrition or malnourished. In addition, as the MNA combines data on nutritional status with clinical observations, disease status and/or laboratory values, it is qualified as an assessment tool too [64]. A shorter version of the MNA was designed, the Mini Nutritional Assessment-Short Form (MNA-SF), to shorten the time needed for its completion [70]. The MNA-SF is a quicker alternative and as effective as the MNA to screen older inpatients [64,70]. Hence, it has been proposed to first screen inpatients using the short version, and if risk of malnutrition or malnutrition is detected the longer version should be performed for its assessment [67]. Indeed, for older adults the European Society for Clinical Nutrition and Metabolism (ESPEN) recommended either of the two versions and proposed that the MNA could be used to ease the nutritional assessment procedure [71].

1.3.2 Adverse consequences of hospitalization on muscle mass and physical function

Although muscle mass loss is an inevitable process of aging, it can be accelerated by acute periods of inactivity or bed rest associated with hospitalization and/or injury or disease [72,73]. Illustration 4 shows how sporadic bouts of muscle disuse affect to muscle loss. Kortebein et al. [74] demonstrated that 10 days of bed rest resulted in a large loss of muscle mass, particularly from the lower extremities, despite being healthy older adults. Similarly, Coker et al. [75] showed that after 10 days of bed rest muscle mass loss was accompanied by a concomitant reduction in muscle strength and physical function in older healthy adults. Thus, these studies reinforced the idea that bed rest along with the physiological stress and other detrimental factor associated with hospitalization might result in further loss of muscle mass and function within older adults during hospitalization [74,75].
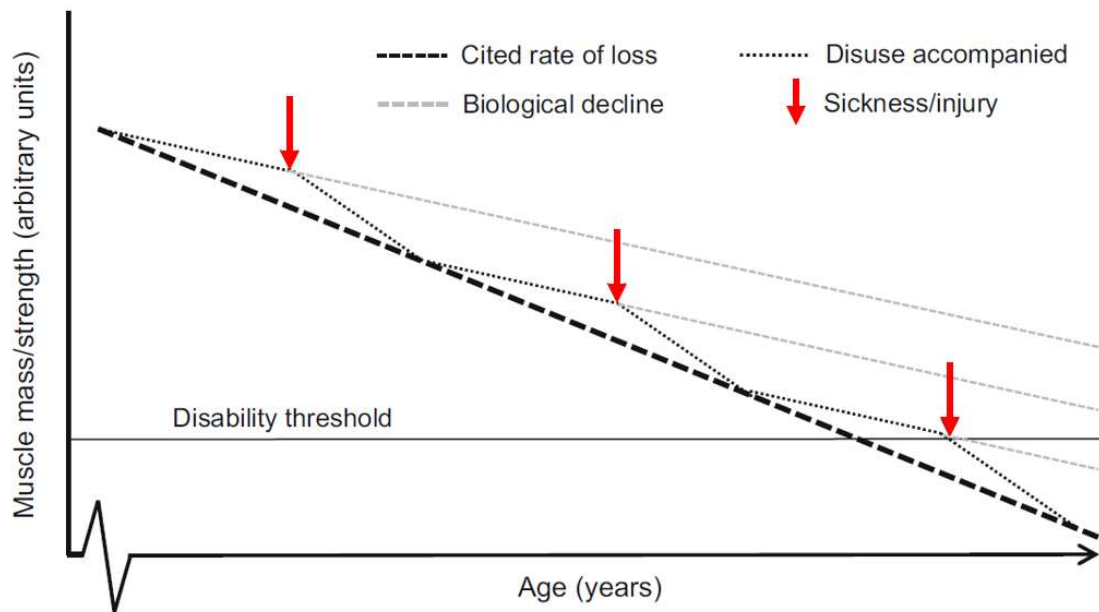
Illustration 4. The decline in muscle mass and strength with advancing age. This figure has been designed to highlight the debilitating impact that sporadic bouts of muscle disuse have on rates of muscle loss. The solid black line is the disability threshold; the broken black line is the mean rate of decline, the broken grey line is the biological decline and the black dotted line is the dis-use accompanied decline. The red arrow refers to a period of disuse or reduced physical activity. Adapted from Witard et al. [73].

Bed rest or limited activity during hospitalization is common among older inpatients [72,76]. Brown et al. [76] showed that, although most of the participants in their study had out-of-bed activity orders, the cohort spent 83.3% of their time lying in the bed, and 13% and 4% of the hospital stay was spent sitting and standing or walking, respectively. Muscle atrophy due to disuse is more pronounced within the first few days of inactivity [72], just coinciding when older inpatients are usually less active [76], negatively impacting clinical outcomes. Likewise, it has been proposed that older adults might benefit from a shorter hospital stay to avoid the detrimental effect on physical function [77]. It has been shown that low mobility is associated with decline in short- and long-term outcomes even within those older adults with stable functional status before being admitted to hospital [78,79]. Furthermore, low mobility was shown to be an independent predictor of poor hospital outcomes despite illness severity, especially regarding the ability to perform activities of daily living which might result in greater risk for dependency at discharge or mortality [79].

This cycle of decline in physical function is also the mechanism underpinning sarcopenia and frailty [80]. As a result, both conditions are common within older hospitalized adults worsening their prognostic [81–84]. Indeed, sarcopenia and frailty are associated with poor

health outcomes [43,81], increase length of hospital stay [81,85] as well as risk for readmission [81,86], and mortality [43,83,86].

Thus, identifying those older inpatients with decline muscle mass, strength and physical function is of utmost importance to ameliorate the negative consequences of hospitalization. Hence, successive short periods of physical inactivity throughout the lifespan might contribute to the age-related loss of muscle mass [72], as many older adults might not fully recovered from the detrimental effects of those brief periods of muscle disuse [87]. This has important clinical implications, as it has been suggested that many older adults might not return to their level at which they can live independently [88].

1.3.2.1 Tools to identify at risk inpatients in clinical settings

There are many methods to measure muscle mass as summarized by Deutz et al. [15]. Those techniques have many advantages and disadvantages [15,89]. The Dual energy X-ray absorptiometry (DXA), despite many limitations, has been proposed as the current reference method to assess muscle mass and body composition in research and clinical setting [15,89]. But overall, the use of these techniques is not affordable for most clinical settings, not only because of their cost but also because of the time and trained personnel needed for their correct use and, therefore are not practical within the daily hospital routine [15]. Likewise, performance-based physical tests are being proposed as surrogate measurements of muscle mass [15].

Physical performance tests might be available even at the most resource-limited settings and are easy-to-use tools by any health-care professional with minimal training needed for their application [15]. As it has been mentioned before, muscle quality (muscle function) might have relatively more functional relevance than muscle quantity (muscle mass), showing a mismatch in the rates of change involving muscle quality parameters and muscle mass measurements [90]. In addition, the rate of decline in muscle strength it seems to be much more rapid than the concomitant muscle mass loss [34]. Also, muscle power might be more important than muscle strength in determining the ability to perform daily activities [37]. Nevertheless, lower muscle mass, strength and power result in lower physical performance which is reflected in lower physical function within older adults. Thus, it would not be surprising if the use of physical performance tests will gain more importance in clinical settings to screen older adults with decline muscle mass, and therefore determine risk of functional limitations. These performance-based physical tests are used to objectively assess physical function [80,91]. Physical function assessment seems crucial for evaluating aspects of health as well as the pathway to disability in

older adults [92]. Several performance-based physical tests such as, handgrip strength and the SPPB, have also shown good validity for predicting poor health outcomes [93,94].

Handgrip strength shows a high prognostic value for predicting physical function [95], comorbidity [96], hospital length [97] and mortality [98,99], and thereby it has been proposed as a biomarker for health status in older adults [95]. Hence, Kaegi-Braun et al. [100] showed a significant association between handgrip strength and the degree of malnutrition in hospitalized older adults. Due to its simple assessment and easy adaptation to almost every inpatient (bedridden or not) handgrip strength is one of the most affordable and easy-to-use tools in clinical settings [15,101,102]. Hence, handgrip strength measured cross-sectionally predicts future function and changes in function over time [102]. In an adult cohort followed for 25 years, those with the lowest baseline grip strength were significantly more likely to have impaired physical function at follow-up [103]. Similarly, Dodds et al. [104] showed that handgrip strength in 'mid-life' predicted mobility and/or personal care disability in early old age.

However, handgrip strength does not reflect overall strength and thereby it has been suggested to be used in combination with lower limb strength measurements [105]. Furthermore, it has been suggested that upper and lower limbs are affected differently by the aging process. It seems that muscle mass and strength are lost more rapidly in the lower limbs [31]. The muscles in the lower body are required for most common activities, and therefore it could be speculated that physical performance tests measuring lower limbs' muscle strength and function might be better predictors of physical function than upper-limbs measurements. In this regard, the Short Physical Performance Battery (SPPB) seems a useful tool. A recent meta-analysis showed that lower extremity physical performance measured by the SPPB (a difference in score of 1 point) was associated with disability to perform activities of daily living [106]. In addition to disability and poor physical function[107], the SPPB has also been shown to be predictive of increased risk of falling in-hospital [108], hospital re-admission [107], increased hospital length of stay [109] and mortality [107,110,111]. Likewise, the SPPB has also gained attention, as it is widely used in clinical settings for physical performance assessment within older adult population [112]. Although, the SPPB is also an affordable and easy-to-use tool, it has some disadvantages in comparison to handgrip strength: the SPPB is not feasible for bedridden inpatients, more space and time is needed to perform this test, and the health-care professional might need to be trained to adequately follow the standardized protocol [113]. In any case, the SPPB is based on 3 tests [113] and each of them were independently related to the physical function of older adults [114], but specially two of them, the 5 times sit-to-stand and

the gait speed tests, have been shown to predict adverse clinical outcomes [106]. Thus, when possible, the implementation of this battery into the daily hospital routine might worth it.

Reference values and general normative data by age groups for handgrip strength [115–117] and the SPPB [118,119] have been published for older adults' population. Thus, it is of clinical and public health interest to feature the physical status of the older hospitalized patients in comparison to their healthy counterparts [95,102]. This will add further clinical information to track the overall health status of patients and to design intervention programs in order to maintain and/or improve muscle mass and strength, and therefore the physical function of older in- and/or post-hospitalized adults.

1.3.3 Malnutrition, sarcopenia, and frailty overlap in hospitalized older adults

According to a recent meta-analysis, it was shown that approximately half of the hospitalized older adults suffer from at least two of these weaken conditions [58]. Thus, malnutrition, sarcopenia and frailty often interact and coexist in older adults [21]. These three conditions share some same characteristic for their diagnostic criteria, such as weight loss, muscle mass and strength loss, and the three conditions in combination and/or independently contribute to a state of greater vulnerability in older adults [58]. So, identifying these conditions among hospitalized older adults is of great interest to prevent and/or manage further complications. However, in clinical settings, the assessment of these conditions is challenging as health-care professionals must cope with many other health conditions and thereby malnutrition, sarcopenia and frailty are often overlooked. Thus, to overcome this time and space limitation often seen in clinical settings, it seems of utmost importance to identify tests that might help on the screening of more than one condition. The early identification of either of the three conditions will help to prevent complications, to better monitor older inpatients' evolution during and after hospitalization and will also permit to design adequate lifestyle interventions based on nutritional support and exercise programs.

1.4 Lifestyle program to counteract the hospitalization effects on malnutrition, sarcopenia and frailty

Hospitalization is a period of high physical stress due to acute and/or chronic illness, where the inpatients experience multiple changes often leading them to a higher vulnerability [120]. This is more exacerbated among older inpatients who are at risk for conditions related to muscle mass and strength loss as well as poor physical function due to an impaired physical reserve associated with aging. Likewise, many of these conditions, such as malnutrition, sarcopenia and frailty might appear or worsen during hospitalization. A consequence that all of

them have in common is the loss of muscle mass and function, and this decline is exacerbated by loss of appetite, inactivity and systemic inflammation occurring during hospitalization [120]. As a result, after a hospitalization an acquired, transient period of vulnerability has been described known as 'post-hospital syndrome', where inpatients experience a period of generalized risk for a range of adverse health outcome [121]. Thus, there is growing interest in early interventions to accelerate recovery and avoid hospital readmission [122]. Interventions that combine nutrition and physical exercise immediately after discharge are suggested as the best choice [122].

1.4.1 Nutritional interventions and protein supplementation

A positive net protein balance, which is considered as muscle protein synthesis exceeding muscle protein breakdown, is important for muscle mass accretion and maintenance [123]. In this regard, energy, but more importantly protein intake are key nutritional factors favouring a positive anabolic response [14,124]. However, it is known that older adults often fail to meet energy and protein requirements as they tend to reduce food intake [12]. It has been often argued that older adults might have a greater requirement for protein and thereby can benefit from protein intakes higher than the current Recommended Dietary Allowance (RDA) (0.8g of protein/kg/day) [125]. Studies aiming to examine the association between dietary protein intake and muscle mass [126] and physical function [127] have shown that older adults with protein intakes above the RDA showed better muscle mass parameters and performed better on the functional tests. Likewise, guidelines for healthy aging focused on protein recommendations, suggest increasing protein intakes to 1.0-1.5g of protein/kg/day [14,125,128], and up to 2.0g of protein/kg/day for older adults with severe illness, injury or with marked malnutrition [128].
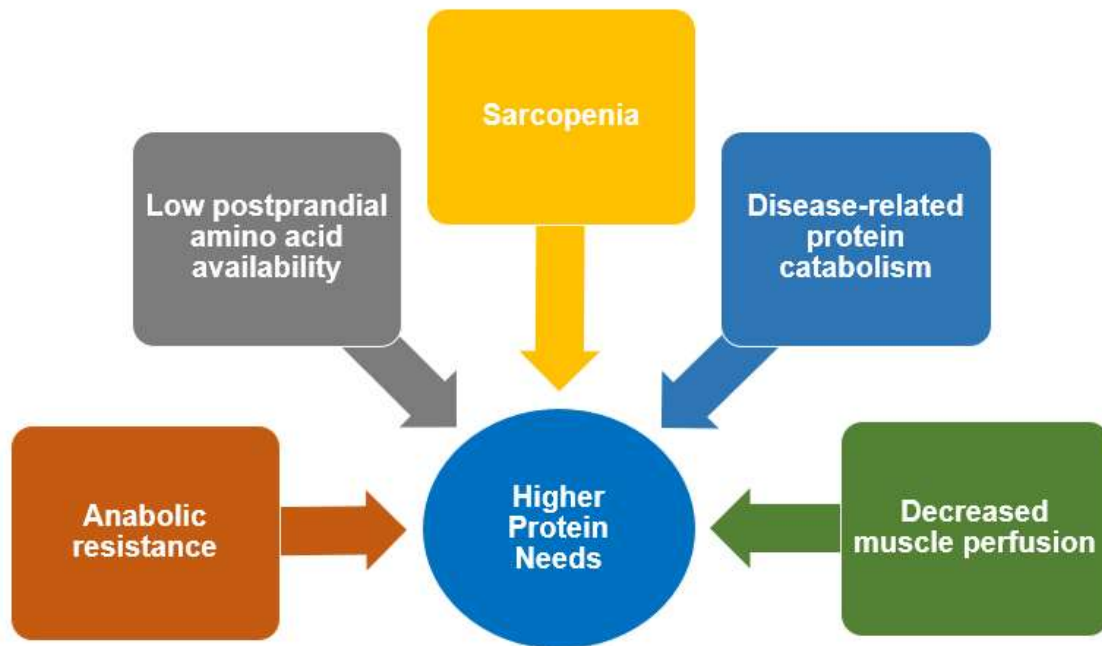
Illustration 5. Factors leading to higher protein needs in older persons. Adapted from Deutz et al. [14].

Simplified, following dietary protein ingestion, protein is digested, and amino acids are absorbed, the rate at which all these occur might determine the postprandial rise in circulating amino acids and thereby their availability for the following uptake by the muscle mass for muscle protein synthesis. Thus, besides total daily protein intake [129], dietary protein quality and its anabolic potential [129,130] have also received increased interest with the goal of optimizing skeletal muscle anabolism in older adults [129].

Dietary protein quality depends on its digestibility, amino acid (AA) profile and AA availability [131]. Likewise, in studies aiming to optimize muscle mass among older adults, whey protein (at least 20 g/day) is considered superior to other isolated protein sources [132]. In addition to its faster digestion and absorption kinetics, whey protein is also known for its high leucine content [133]. Leucine is the main precursor for activating muscle protein synthesis via mammalian target of rapamycin (mTOR) signalling [123,134]. A protein source containing around 1.8-2.0g of leucine would be enough to activate post-exercise 'leucine trigger', whereas in rested conditions a higher dose might be required [134]. In older adults a greater 'leucine threshold' have been defined requiring higher levels of protein/leucine [135]. However, considering the low appetite and thereby the low energy intake in older adults, it has been suggested that lower protein doses with higher quality proteins, such as whey protein, might help to achieve the 'leucine threshold' [135].

In addition, it has been observed that with aging there is a reduced sensitivity of muscle mass to anabolic stimuli, such as feeding and muscle contraction, resulting in a blunted muscle

protein synthetic response to those anabolic stimuli [123]. This is known as age-related anabolic resistance, and it can be overcome by the synergistic effect of both, protein supplementation and resistance exercise [123,134]. Likewise, another proposed strategy to stimulate muscle protein synthesis has been to supplement a leucine-enriched protein dose just after finishing a resistance training session to take advantage of the greater sensitivity to anabolism induced by muscle contraction [135]. Luiking et al. [132] reported that 20g of whey protein enriched with 3g of leucine post-exercise resulted in a greater muscle protein synthesis rate in healthy older adults.

It is not completely understood what causes the age-related anabolic resistance [123]. Impairments in several physiological processes have been suggested to explain it, such as reduced rate of dietary digestion, amino acid absorption and/or a greater splanchnic amino acid retention [123,136]. Another plausible theory is the gradual decline in physical activity with aging as well as the physical inactivity *per se* [123]. Likewise, the commonly known expression 'use it or lose it' seems to perfectly reflect the interrelation between physical inactivity and muscle loss with disuse regardless of age or disease state [137]. In a study carried out by Breen et al. [138] it was shown that a decrease of daily activity (decreased step count) had substantial effects on the anabolic capacity of the muscle. Thus, it is well-stablished that muscle contraction plays an important role in the sensitivity of old muscle to anabolic factors such as dietary protein/Aa [73,135,137]. In this regard, resistance type exercises seem to be the greatest stimulus to increase this sensitivity to protein ingestion in older adults [73,135]. However, controversial findings have been reported in older adults, with some showing significant improvements after post-exercise protein supplementation [139,140], whereas others did not show further significant benefits with protein supplementation after resistance training [141].

1.4.2 Exercise interventions

Resistance training is now widely recognized for its many health benefits, being considered 'the most potent non-pharmacological stimulus to increase skeletal muscle mass, strength and improve physical function' [142]. Ample gains in whole body muscle mass and strength have been reported after 12-24 weeks of structured resistance training program in older adults [143–145]. More recently, Kirk et al. [146] showed that 16 weeks of resistance training plus functional (3 times/week) exercises significantly increased muscle strength, physical function, and aerobic capacity. Hence, Churchward-Venne et al. [147] demonstrated that even the very old individuals maintain the capacity to increase muscle fiber size in response to resistance training and that despite interindividual variability in the adaptive response, there

was not any single participants that did not positively respond to this type of training. Thus, these results highlight that the anabolic effects of resistance training on muscle mass and strength are not restricted by older adults' age, and thereby this type of training should be promoted to counteract age-related muscle mass and strength loss [147].

Nowadays, resistance type exercise training is considered the most effective countermeasure to support healthy aging of muscle mass, and, although there is no a robust consensus yet, it appears that these benefits might increase along with post-exercise protein supplementation [148]. Likewise, current guidelines to prevent and/or counteract sarcopenia [142,149], frailty [150] and other conditions that also derived in muscle weakness, such as malnutrition [51] or age-related chronic diseases [151], aligned with these recommendations.

1.4.2.1 Role of myokines

Resistance training provides the necessary stimulus to promote muscle hypertrophy through several signalling pathways [152]. Muscle contraction triggers the release of myokines that influence those signalling pathways and thereby muscle growth [153]. Unlike follistatin and irisin, myostatin's release is downregulated by exercising muscles [153,154]. Thus, myostatin is known as a negative regulator of muscle mass [154]. In contrast, the release of follistatin and irisin by muscle is supposed to be increased in response to exercise [155–159], and therefore both myokines have been associated with muscle mass growth [153,160,161].

Recent studies have examined how myokines act in response to exercise training in older adults to see whether the obtained benefits could be mediated by myokines' responses or not. Hofmann et al. concluded that the improvements observed after resistance training program could have been mediated by follistatin induces blocking of the muscle degradation pathways rather than by lower circulating levels of myostatin [162]. This study also highlights the importance to not only measure single molecules, but also their interaction, such as the follistatin to myostatin ratio [162,163]. Nevertheless, there is still much controversy regarding the response of both myokines to exercise, but especially according to myostatin, some reporting a reduction [164,165] while other showing a higher myostatin concentrations after an exercise intervention program [163,166]. Regarding irisin response to exercise training, it seems that aerobic and resistance training [156,159,167] stimulate irisin release increasing its circulating levels in older adults, but there are studies showing no changes too [168].

There is growing interest to know if those myokines might be contributing to the muscle weakness seen with aging [169], and to see if they could serve as blood-based biomarkers

[170,171]. However, there are still many aspects that need to be studied before, such as myokines' dynamic in response to exercise and aging.

2. SUBJECTS AND METHODS

This doctoral thesis was carried out in a clinical setting, specifically at the facilities of the Araba University Hospital in Vitoria-Gasteiz from September 2017 to July 2018. The Clinical Research Ethics Committee of the Araba University Hospital (CEIC-HUA: 2017-021) approved the protocol to proceed with the Sarcopenia and Fragilidad-protein (S and F-PROT) study. As a result, this doctoral thesis is based on two studies: a) a cross-sectional study to investigate the nutritional status and physical function in hospitalized older adults (≥70 years old), and b) a randomized controlled trial in post-hospitalized older adults (≥70 years old) to examine the effects of 12-weeks of resistance training program along with leucine-enriched whey protein supplementation on muscle mass, physical function, and sarcopenia and frailty statuses (ClinicalTrials.gov ID: NCT03815201). From the cross-sectional study two different scientific works were carried out **(Study 1 and 2)**, and two else from the randomized controlled trial **(Study 3 and 4)**.

2.1 Malnutrition and physical function in hospitalized older adults

2.1.1 Design: Participants and data collection

Participants were older inpatients at the internal medicine service of the Araba University Hospital. Members of the research team, with a wide experience in clinical settings, revisited the daily list of patients admitted to the internal service as well as the medical history by revising the clinical records to assess eligibility. Patients meeting the following criteria were eligible for inclusion and were evaluated within the first 3 days upon admission: age ≥70 years old, cut-off at the Mini Mental State Questionnaire ≥20, to be able to walk alone or using assistive devices (walking stick or walking frame), to be able to understand and follow the instructions, and agreed to participate in the study and signed the informed consent. However, they were not eligible for evaluation if they had any of the following exclusion criteria: been suffering from severe dementia or Parkinson, been unable to stand and/or walk a short distance, been in critical medical condition (e.g., need of palliative care and/or advance cancer) or death, and if they had suffered any fracture of the upper or lower limbs in the last 3 months.

A total of 1878 were assessed and from them 604 participants were included in the Study.

2.1.2 Measurements

2.1.2.1 Nutritional assessment

Nutritional status of the older inpatients was assessed by the MNA-SF (Nestlé Nutrition Institute, [172] questionnaire directly with patients and/or their respective relatives or

caregivers. This questionnaire compromises 6-items and each answer has a numerical value contributing to the final score [172]. A maximum of 14 points can be obtained and depending on the score, the following categories are described: 0-7 points malnutrition, 8-11 points at risk of malnutrition and 12-14 points normal nutritional status [172]. The last item of the MNA-SF can be answered by body mass index estimation or by measuring the calf circumference [172]. Due to difficulties measuring height in several patients, it was decided to use calf circumference following the standard protocol recommended by the International Society for the Advancement of Kinanthropometry. When possible, body mass (kg) was measured barefoot based on standardized protocols (OMROM HN-288, Digital Personal Scale, Barcelona, Spain).

For several analyses, the three MNA-SF categories were re-coded into two categories. Those at risk of malnutrition or malnourished were grouped together into 'malnutrition or risk of malnutrition' as both are considered risk factors within older adult population and the remaining category was 'normal nutritional status'.

2.1.2.2 Physical function assessment

Physical function was assessed using two tests: handgrip strength and the SPPB. Dominant handgrip strength (kg) was measured by a handheld dynamometer (JAMAR® PLUS + Hand Dynamometer) in a seating position, as it has been proposed for older adults in clinical practice [173].

The SPPB clinical tool was chosen to measure physical function of the lower limbs [113]. The SPPB assessment methodology has been published elsewhere [113] and includes 3 subtests: 1) the standing balance test, 2) the gait speed test and 3) the 5 times sit-to-stand test. The total SPPB score ranges from 0 to 12, with higher scores reflecting better functional status, and it is divided into 4 clinically relevant categories: from 0 to 3, from 4 to 6, from 7 to 9 and from 10 to 12 points [113].

2.1.2.3 Comorbidity risk

Comorbidity burden was defined according to the Charlson Comorbidity Index (CCI) [174]. The estimation of this index is based on age (divided into 5 ranges) and 17 different categories of comorbidity [174]. Each age range and category have an associated score (from 1 to 6, the latter based on the severity of the condition), and then all are summed contributing to the total score [174]. Thereafter, 3 different categories are defined to classify comorbidity risk within patients: 1) 1-2 points mild risk, 2) 3-4 points moderate risk, and 3) ≥ 5 points severe risk [174].
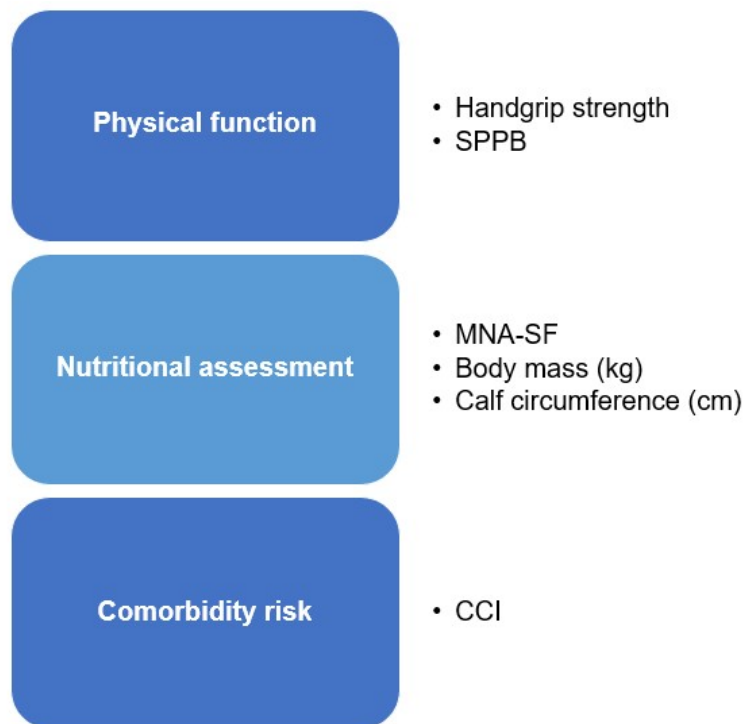
Illustration 6. Summary of the variables and measurements in the cross-sectional study. Abbreviation: SPPB: Short Physical Performance Battery; MNA-SF: Mini Nutritional Assessment-Short Form; CCI: Charlson Comorbidity Index.

2.2 Effects of a resistance training program along with leucine-enriched protein supplementation in post-hospitalized older adults

2.2.1 Design: participants and data collection

The same inclusion criteria as for the cross-sectional study were followed for the randomized controlled trial. According to exclusion criteria, beside the same exclusion criteria as for the cross-sectional study, patients were excluded if they had: history of chronic kidney disease, had suffered a heart attack in the last 3 months, history of autoimmune neuromuscular disorders (for example, myasthenia gravis, Guillain-Barré syndrome, inflammatory myopathies) or amyotrophic lateral sclerosis. Patients that were eligible for the intervention were informed about the possibility of participating in an exercise training program after hospital discharge and an informed consent was given along with further written information. After a recovery week, patients were cited for baseline physical function assessment before initiating the intervention program. However, as the hospital recruitment proved not to be enough for the intervention aims, the outpatient internal medicine service was chosen as an alternative recruitment source. The doctor informed those patients potentially meeting inclusion criteria at the outpatient internal medicine service about the exercise intervention program. Thereafter, patients were

cited for a first eligibility assessment with the investigation team. If participation criteria were met, patients were again cited a week after for baseline physical function.

Finally, a total of 41 participants were randomized and from them, 28 finished the intervention program.

2.2.1.1 Randomization

Following baseline physical assessment, participants were randomly assigned in a parallel design (1:1 ratio, stratified by gender) to one of the two intervention groups: Placebo-group or Protein-group. Randomization was conducted by the researchers involved in the intervention, so blinding was not possible for them.

2.2.1.2 Supplementation and blinding

Placebo and protein supplements were delivered by the nutritionist in the first half hour following each training session. The protein supplement contained 20g of whey protein isolate (Davisco®: BiPRO all-natural whey protein isolate, Eden Paririe, MN, USA) enriched with 3g of leucine (Nutricia, Madrid, Spain). The nutritional composition of both the placebo and protein supplement is shown in table 1. The supplements were energy-matched and flavoured with lemon flavor and solubilized in 150mL of water. Only participants were blinded for supplementation. Supplements were stored in boxes and only the research team could identify them. All supplements were developed, prepared, and stored in boxes by Laboratorium Sanitatis SL (Tecnalia Research and Innovation, Vitoria-Gasteiz, Spain).

Table 1. Nutritional composition of the protein and placebo supplements.

Nutritional composition	Protein supplement
B-lactoglobulin (g/bottle)	20
L-Leucine (g/bottle)	3
Sodium saccharin (g/bottle)	0.050
Sucralose (g/bottle)	0.030
Lemon flavour 654500 (g/bottle)	0.250
	Placebo supplement
Maltodextrin (g/bottle)	23
Hydroxyethylcellulose (g/bottle)	0.200
Lemon flavour 654500 (g/bottle)	0.250

2.2.1.3 Design of the resistance training program

Both groups followed a supervised resistance training program for 12 weeks. The program consisted of one-hour sessions on two non-consecutive days per week. The first week of intervention was used for familiarization, and 1-RM (repetition maximum) estimation by the individual's functional capacity through Brzycki equation [175]. The loads were then gradually increased during a month, and half exercises were performed at 50%-65% of the estimated 1-

RM. During the subsequent months, load was increased until 70% of the estimated 1-RM was reached. Two sets were performed per exercise and load and maximum repetition for each exercise was personalized for each participant. All resistance training sessions were designed and supervised by a sport scientist with experience in resistance training for the elderly.

All training sessions started with warm-up exercises and were followed by strengthening exercises of upper and lower limbs. In the same resistance training session, some exercises for dynamic balance improvement were also practiced. The session finished with five minutes of cool-down, consisting mainly of stretching exercises.

2.2.2 Measurements

All measurements were performed at baseline and after 12 weeks of intervention (at week 13) by the same trained researchers. Post-intervention measurements were scheduled within one week following the last exercise session.

2.2.2.1 Physical function

Physical function was assessed using a combination of tests. The tests used to assess lower and upper body strength and aerobic capacity were based on the Senior Fitness test [176]. For lower and upper body strength, 30-Second Chair Stand Test and 30-Second Arm Curl Test were used, respectively. For upper body strength, isometric handgrip strength was also measured using a handheld dynamometer (same as cross-sectional study). The test was performed twice, alternating each hand; the highest value was chosen and used for analysis. Aerobic capacity was assessed by the six minutes walking test. Hence, for physical function assessment, the SPPB test battery was also used as described for the cross-sectional study.

2.2.2.2 Nutritional assessment and body composition

A nutritionist completed all nutritional questionnaires along with the participant and/or participant's relative or caregiver. Participant's nutritional status was assessed using the long version of the MNA questionnaire (Nestlé Nutritional Institute) [69]. This questionnaire contains 18 items divided into four categories: anthropometric assessment, general assessment, short dietary assessment and subjective assessment [69]. Each answer has a numerical value contributing to the final punctuation. A maximum of 30 points can be obtained. Punctuation ranging from 24 to 30 reflects normal nutritional status, from 17 to 23.5 risk of malnutrition and a punctuation under 17 reflects malnutrition [69].

Body fat, lean mass, fat-free mass, bone mass, bone mineral density and bone mineral content were assessed by dual-energy X-ray absorptiometry (DXA; HOLOGIC, QDR 4500,

Bedford, MA, USA). Likewise, the sum of lean mass from both arms and legs was used to assess appendicular skeletal muscle mass (kg) and this was divided by height squared to assess Appendicular Skeletal Muscle Mass Index (kg/m^2). Similarly, the Fat Mass Index was calculated as the total fat mass divided by height squared (kg/m^2) and Fat-Free Mass Index as total fat free mass divided by height squared (kg/m^2).

Body mass (kg) (OMROM HN-288, Digital Personal Scale, Kyoto, Japan) was measured barefoot following the standard protocols. Height was estimated using knee height determination (SECA 220, Hamburg, Germany) [177]. Body mass index was calculated as body weight divided by height squared (kg/m^2). Waist circumference, hip circumference and mid-arm circumference were measured with a nonelastic tape (CESCORF, Rio Grande do Sul, Brazil) following the protocol recommended by the International Society for the Advancement of Kinanthropometry (ISAK). Calf circumference was measured with the same nonelastic tape on the left side following the instructions of the MNA questionnaire [69]. All measures were performed twice and the average was used for analysis.

2.2.2.3 Sarcopenia and frailty assessment

Sarcopenia was assessed following the proposed algorithm for finding cases by the EWGSOP2 in the revised European consensus on the definition and diagnosis of sarcopenia [38]. For this randomized controlled trial participants were first screened for muscle strength according to the handgrip strength cut-off points, and those with low muscle strength were then assessed for muscle quantity based on appendicular skeletal muscle mass (kg) cut-off points to confirm sarcopenia [38].

The SPPB threshold was used for frailty assessment in this randomized controlled trial [178,179].

2.2.2.4 Blood-based biomarkers

Biochemical parameters were obtained from fasting venous blood samples in Ethylenediaminetetraacetic acid-containing tubes and in serum tubes a week after the last training session. These tubes were immediately carried to the laboratory and EDTA-containing tubes were centrifuged at 1000 x g at 4 ºC for 10 minutes, whereas serum tubes were centrifuged 90 minutes after blood collection at 1000 x g at 20 ºC for 15 minutes. For the measurement of biomarkers, the quantification was carried out by spectrophotometry with FLUOstar OPTIMA Microplate reader (ThermoFisher Scientific, Waltham, MA, USA) and Optima

Control software version 2.20 (BMG, LABTECH, Ortenberg, Germany). Serum albumin, prealbumin and creatinine were measured as protein malnutrition markers.

The obtained serum aliquots from the participants were stored at -80 ºC for further analyses. Myokine concentrations were quantified by commercial enzyme-linked immunosorbent assays (ELISA), following the manufacturer's protocol. Serum myostatin (ng/ml) and follistatin (ng/ml) were measured using GDF-8/Myostatin and Follistatin Quantikine ELISA kits, respectively (R&D Systems Inc., Minneapolis, MN, USA). Serum irisin (µg/ml) concentration was measured using an Irisin ELISA kit (AdipoGen LifeSciences, San Diego, CA, USA).

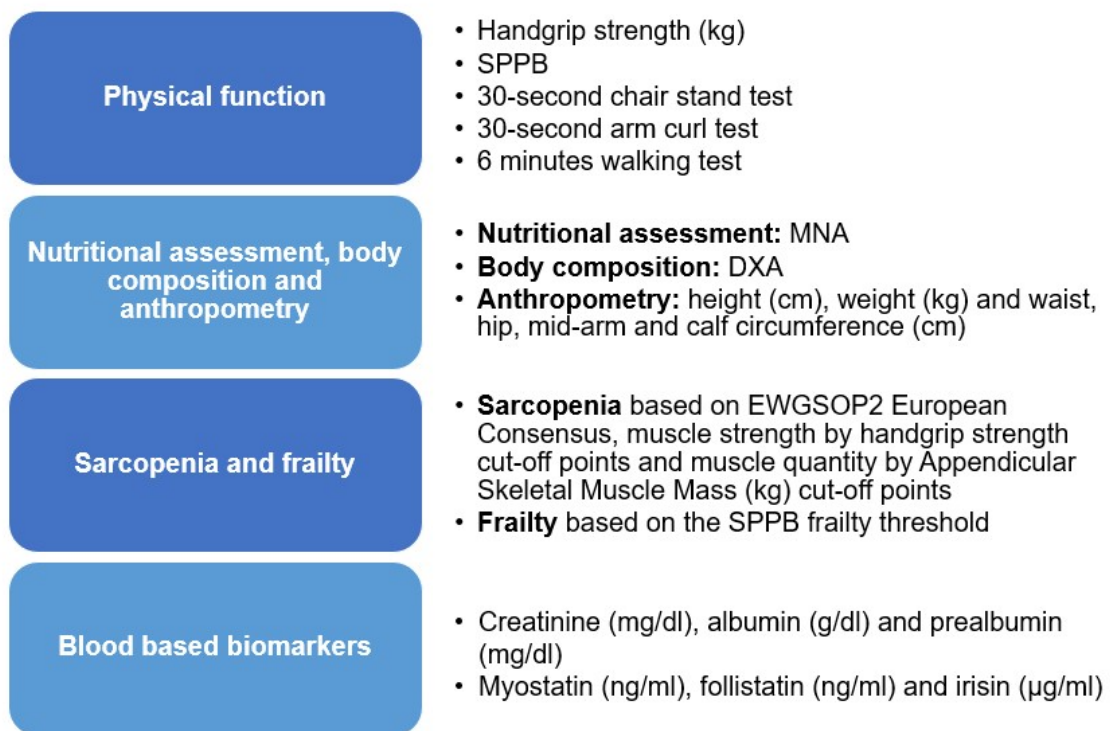| **Physical function**	• Handgrip strength (kg) • SPPB • 30-second chair stand test • 30-second arm curl test • 6 minutes walking test
Nutritional assessment, body composition and anthropometry	• **Nutritional assessment:** MNA • **Body composition:** DXA • **Anthropometry:** height (cm), weight (kg) and waist, hip, mid-arm and calf circumference (cm)
Sarcopenia and frailty	• **Sarcopenia** based on EWGSOP2 European Consensus, muscle strength by handgrip strength cut-off points and muscle quantity by Appendicular Skeletal Muscle Mass (kg) cut-off points • **Frailty** based on the SPPB frailty threshold
Blood based biomarkers	• Creatinine (mg/dl), albumin (g/dl) and prealbumin (mg/dl) • Myostatin (ng/ml), follistatin (ng/ml) and irisin (µg/ml)

Illustration 7. Summary of the variables and measurements in the randomized controlled trial. Abbreviations: SPPB: Short Physical Performance Battery; MNA: Mini Nutritional Assessment; DXA: Dual-energy X-ray absorptiometry; EWGSOP2: European Working Group on Sarcopenia in Older People 2.

3. HYPOTHESIS AND OBJECTIVES

3.1 Hypothesis

The hypothesis of the current International Doctoral Thesis are:

I.   A resistance training program along with leucine-enriched protein supplementation immediately after discharge will improve or even reverse the detrimental effects of hospitalization on nutritional status and physical function in older adults.

II. Those older adults with sarcopenia and/or frailty might benefit most from this nutritional and exercise intervention, and those improvements might be highlighted by changes in plasma myokine concentrations.

3.2 Main objectives

To test our hypothesis, we first contextualized the nutritional and physical status, and the associated comorbidity risk in older hospitalized adults at the internal medicine service of the Araba University Hospital in Vitoria-Gasteiz (Spain), and thereby justify the importance of a resistance training program and protein supplementation immediately after hospitalization. For this aim we carried out a cross-sectional study that aimed:

I.   To describe the nutritional and physical status and characterize the physical functional status of hospitalized older adults aged ≥ 70 years old. **Study 1.**

II. To analyse the association between the Charlson Comorbidity Index (CCI) with nutritional status and physical function of hospitalized older adults aged ≥ 70 years old. **Study 2.**

Then, in order to test the hypothesis, a randomized controlled trial was designed aiming:

III. To examine the effects of a resistance training program along with whey protein supplementation enriched with leucine post-exercise on muscle mass and strength gains in a post-hospitalized older adults' population aged ≥ 70 years old. **Study 3.**

IV. To examine the effects of a resistance training program along with post-exercise leucine-enriched whey protein supplementation on sarcopenia and frailty status in a post-hospitalized older adults' population aged ≥ 70 years old. **Study 4.**

Secondary objectives:

Some secondary objectives resulted from the cross-sectional study and from the randomized controlled trial:

I.   To examine the association between malnutrition and physical functional status of the studied sample. **Study 1.**

II. To evaluate the utility of physical performance tests on malnutrition management, and *vice versa* in hospitalized older adults aged ≥ 70 years old. **Study 1.**

III. To investigate the individual and combined associations of nutritional status assessed by the MNA-SF, fitness status according to handgrip strength, and frailty status assessed by the SPPB frailty threshold of older inpatients aged ≥ 70 years old with comorbidity risk (CCI). **Study 2.**

IV. To analyse the effects of a resistance training program with post-exercise enriched protein supplementation on plasma myokine concentrations of post-hospitalized older adults aged ≥ 70 years old. **Study 4.**

4. SUMMARY AND DISCUSSION

4.1 Nutritional status and physical function of hospitalized older adults

The first two studies in this doctoral thesis (**Studies 1** and **2**) were cross-sectional secondary analyses conducted as part of the recruitment for a randomized controlled trial (ClinicalTrials.gov ID: NCT03815201) at the internal medicine service of the Araba University Hospital in Vitoria-Gasteiz (Spain). These studies aimed to examine the nutritional status and physical function of hospitalized older adults. Specifically, the **Study 1** aimed to characterize the physical condition of hospitalized older adults in comparison to previously published reference percentile values and to examine the association between the nutritional status and the physical function of the studied sample. The **Study 2**, aimed to examine the association of comorbidity with the nutritional status and with the physical function of older inpatients, and to examine whether malnutrition and poor physical function, independently or in combination, are associated with higher comorbidity risk in hospitalized older adults.

4.1.1 In-hospital malnutrition increased the risk for poor functional status in older adults

The main findings of this study were that 1) a high percentage of older hospitalized adults were at risk of malnutrition or malnourished (65.7%), 2) performance worsen along with worst nutritional status, and 3) being at risk of malnutrition or malnourished increased the risk for being classified as unfit for the handgrip test, the SPPB score and the gait speed test, and for being frail according to the SPPB frailty threshold.

The elevated prevalence of malnutrition or risk malnutrition seen in our study is within the range reported by other studies carried in clinical settings using either the full MNA or the MNA-SF [29,30,59,180]. Thus, we confirmed that a high proportion of older adults are already malnourished or at risk for malnutrition during hospitalization among the older adults' population [58]. Hence, the older inpatients in this study showed a poor physical function which fell far below what it would have been considered appropriate for their age. When compared to other studies, our studied sample of older adults showed better [180,181] or worse [182] performance within those tests. These contradictory findings might be due to the size and age-range of the sample in those studies [180–182]. Nevertheless, our results confirm that muscle strength and function decrease with aging and that the decrease seems to be steeper within men [116,183].

To our knowledge the **Study 1** is the first work including a large sample (n = 604) of hospitalized older adults (≥ 70 years old) to examine their nutritional status and its association with the most widely and easy-to-use physical performance tests in clinical settings (handgrip

strength and SPPB). Each of those tests have been examined in prior studies individually in association with nutritional status, but only a few of them were carried out in hospitalized older adults and/or the study sample were smaller and/or included a wide age range in comparison to our study [57,101,102,180,182,184]. As far as we are aware, there is only one study examining the association of both handgrip strength and SPPB, as well as two of its subtests separately with nutritional status, but it was carried in geriatric outpatients and malnutrition was assessed using a different nutritional questionnaire to the one used in the current study [185].

Handgrip strength has been proposed as potentially useful and rapid tool for nutritional assessment in hospital patients [101,182], but the usage of the SPPB and its subtests for this aim has not been studied yet [110]. In this regard, the results from this study might be meaningful for clinical settings as they highlight the negative effects of the nutritional status on muscle function and therefore, on physical performance due to the well-stablished muscle mass loss seen with malnutrition [15]. Hence, our results showed, in agreement with other studies, that the SPPB as well as its subtest, the gait speed test, might have individual value in relation to malnutrition [180,184,185]. However, according to handgrip strength our results contrast with those from hospitalized patients where age was limited to ≥65 years old [57], but it is worth mentioning that the small sample size and the different nutritional screening tools used for malnutrition in those studies might have hidden any comparison.

In relation to frailty, its association with malnutrition has been reported by other studies carried in clinical settings [21,82,84], but those studies have often used frailty criteria (such as, the Frailty Instrument for Primary Care of the Survey of Health, Ageing and Retirement in Europe [82,84] or the Fried Frailty Criteria [21]) containing items shared by the MNA-SF questionnaire, so it is not surprising to have found an association in those studies. Thus, a relevant finding of our study had been that although frailty was assessed by physical performance measurement (SPPB frailty threshold), without subjective questions shared by the MNA-SF, those older inpatients at risk of malnutrition or malnourished had ≥ 4.5 higher risk of being classified as frail according to the SPPB frailty threshold reinforcing the link between malnutrition and physical performance level.

Thus, the **Study 1** brings new insights into malnutrition and poor physical performance management that aligned with recent guidelines [15,112,186]. Although, more studies are needed to confirm our results, our study highlights the use of handgrip strength and SPPB, as well as its subtests, in hospitalized older adults to complement nutritional screening. The MNA-SF is more susceptible to the patient's mood as well as to patient's cognitive and health status

due to its subjectivity, although it can be answered by a family member or a caregiver [70]. Nevertheless, according to our results handgrip strength and the SPPB tests seem to be adequate alternative options as they provide objective information for malnutrition screening and add additional information by identifying those at risk of functional limitations as well as to monitor patient's progress during hospitalization and post-discharge. Hence, it is well-known that malnutrition and poor physical performance as well as frailty are often related and overlapped within older adults along with other age-related syndromes such as, sarcopenia [21]. Thus, identifying tests that might help on the screening of more than one condition seems of great interest due to time-, resource- and/or space-related limitations in many clinical settings. Although, we acknowledge that each condition then needs to be identify by a specific tool aimed for that, according to our study results, depending on the clinical setting and/or older adults' characteristics a test might be chosen for a first step screening.

4.1.2 Relationships between malnutrition, physical function, and comorbidity in hospitalized older adults

As far as we are aware, this is the first study aiming to examine the association between nutritional status assessed by the MNA-SF and physical function assessed by handgrip strength and the SPPB with comorbidity risk according to the CCI. The main findings of the **Study 2** were that 1) those hospitalized older adults aged 70 and older at risk of malnutrition or malnourished and/or with poor physical function had higher values of comorbidity than those with normal nutritional status and/or good physical function. Indeed, 2) those inpatients at risk of malnutrition or malnourished and frail (SPPB frailty threshold), had significantly higher risk of being at severe risk of comorbidity than their peers with normal nutritional status and non-frail (SPPB frailty threshold). In addition, 3) older inpatients classified as non-frail (SPPB frailty threshold) had lower values of CCI regardless of their nutritional status.

In the current study, older hospitalized adults at severe risk of comorbidity were significantly older, had higher rate of polypharmacy and showed higher rates of chronic diseases as well as worse nutritional status and physical function compared to those at moderate risk of comorbidity. Indeed, our results showed that higher punctuation in the MNA-SF was inversely associated with the CCI score among older inpatients, with malnourished older adults showing an increased risk (twofold increased risk) for severe comorbidity risk. This underpins MNA-SF as an adequate tool predicting unfavourable clinical outcomes [187]. Furthermore, the SPPB frailty threshold might be even a better choice, considering that in the current study being frail according to this frailty criteria increased the risk for severe comorbidity almost fourfold to that seen in non-frailty hospitalized older adults. Although, it seems that the current study is in line

with previous studies [188], our results need to be further examined, as none of the studies linking frailty to multimorbidity used the SPPB to assess frailty [188]. Hence, the SPPB has also gained attention due to its ability to predict mortality [110,111]. Taking into account that the CCI is often used to predict mortality, it could be speculated that due to the increased risk for severe comorbidity seen in frailty older inpatients in the current study, the SPPB frailty threshold might help to identify those older inpatients at risk of mortality. However, we failed to show an increased risk for severe comorbidity according to handgrip strength, questioning its ability to predict health negative outcomes as suggested previously [102].

Lastly, an interesting finding from the current study was the synergetic effect observed between the nutritional status and performance-based physical tests. Hospitalized older adults at risk of malnutrition or malnourished had higher CCI scores regardless of being fit or unfit according to handgrip strength, whereas frail patients showed higher CCI scores despite being well-nourished or malnourished. The results in this study suggest that frailty, assessed by the SPPB frailty threshold, might be a major contributor to the CCI increase that the nutritional status in hospitalized older adults. Nevertheless, our results along with a recent study carried out by Tonet et al. [189] in older adults with a specific characteristic (acute coronary syndrome), arise new insights to the use of the MNA-SF and the SPPB in clinical settings for predicting adverse clinical outcomes.

4.2 Lifestyle interventions to counteract muscle mass and strength loss, and sarcopenia and frailty in post-hospitalized older adults

The last two studies in the current Doctoral Thesis were based on a single-blind randomized controlled trial (ClinicalTrials.gov ID: NCT03815201) (**Study 3** and **Study 4**) conducted at the facilities of the Araba University Hospital in Vitoria-Gasteiz (Spain). Both studies examined the effects of a leucine-enriched whey protein supplementation in post-hospitalized older adults, but the **Study 3** aimed to examine the effects on muscle mass and strength gains as well as physical function, whereas the **Study 4** was a secondary analysis that aimed to examine the effects on sarcopenia and frailty status and on plasma myokine concentrations.

4.2.1 Effects of 12-weeks of resistance training intervention along with leucine-enriched whey protein supplementation on physical function in post-hospitalized older adults

To our knowledge, this is the first study including post-hospitalized older adults almost immediately after discharge (~ 1 week) in a supervised resistance training program along with post-exercise leucine-enriched protein supplementation. The main finding was that no

additional beneficial effects were observed with leucine-enriched whey protein post-exercise supplementation after 12 weeks of resistance training (2 sessions/week) on any of the measured variables. Despite this, 12 weeks of resistance training program immediately after discharge proved to be effective in improving physical function of older adults.

Resistance-type exercises are being suggested as the primary and more effective countermeasure for age-related muscle mass and strength loss [73,142], 12-24 weeks of resistance training have been reported to be effective in this regard [143–146,190]. Thus, our results continue to sustain those recommendations. However, our intervention period (12 weeks) was not enough to observe significant changes in muscle mass in either of the intervention groups. It has been suggested that the optimal period to observe effects on muscle mass in healthy older adults is 50-53 weeks, which we were far from achieving [191]. Nevertheless, Churchward-Venne et al. [147] stated that there are older individuals who demonstrate little to no improvements after 12 weeks of training, but show substantial improvements after 24 weeks of training. Thus, if we had lengthened our intervention up to 24 weeks, we might have seen significant improvements in muscle mass. Nevertheless, this optimal period might also vary depending on the intensity and volume of the training sessions, the number of sessions per week as well as older adults' characteristics [192,193].

Furthermore, it has been suggested that the blunted anabolic response observed in older adults might be overcome, or at least minimized, if adequate interventions are designed [73,135,137]. Likewise, in the current study we considered 20g of whey protein supplementation enriched with 3g of leucine as a complementary strategy after each training session (2 sessions/week) to take advantage of the anabolic response induced by resistance training [130,132]. However, no further benefits were seen on physical function for the protein-group. The additional beneficial effects of protein to increase resistance training induced adaptations are not cleared no for muscle strength nor muscle mass, with some showing further benefits [130,144] and others no additional effects [146,190]. Contradictory results have been reported and this might be due to the poor compliance, high heterogeneity, and underpowered studies regarding protein supplementation in older adult population [139–141].

Nevertheless, we hypothesized in our study that a plausible explanation for not having seen further effects with protein enriched supplementation was that participants met, or even were above the RDA, for protein [194]. Otherwise, muscle mass loss should have been seen as participants in the current study were post-hospitalized older adults, who are supposed to be in a transient period of vulnerability requiring an increase dietary protein and energy intake

[121,128]. In contrast, muscle mass did not decrease, it was maintained which is of utmost importance to recover from an acute period of hospitalization in older adults. Hence, this hypothesis was also supported by the observed baseline score (24.5 points, considered normal nutritional status) in the MNA questionnaire for the protein-group. Additionally, the proposed hypothesis might also explain why in comparison to the placebo-group, the protein-group did no show further improvements in physical function. Some authors have suggested that if adequate protein and energy intake occurs in untrained individuals starting a resistance training program, the stimulation provided by this training overcomes the stimulus coming from diet, and thereby trained individuals might benefit more from protein supplementation [190,195,196].

Overall, our results support resistance training as a fundamental early intervention strategy to increase muscle strength and improve physical function in post-hospitalized older adults. Although, significant improvements in muscle mass were not seen, it might be important to highlight that muscle mass was maintained and it might be speculated that if the intervention had been prolonged significant improvements might have been observed.

4.2.2 Effects of 12-weeks of resistance training intervention along with leucine-enriched whey protein supplementation on sarcopenia and frailty in post-hospitalized older adults

The main finding of the current study was that the addition of leucine-enriched whey protein to the resistance training program did not cause any significant improvement to frailty and sarcopenia status. Hence, no differences between groups were observed in blood-based biomarker analyses.

Resistance training is currently recognized as the most effective strategy to counteract sarcopenia [142,149,197] and frailty [150] in older adults. The potential of resistance training to positively influence muscle strength and thereby physical function in sarcopenic [146,195] and frail [144,196] older adults has been widely shown, whereas improvements related to muscle mass measurements are contradictory [197,198]. As stated in the Study 3 the intervention period as well as the design of the resistance training program seem important factors determining the achievement of significant positive results in terms of muscle mass in older adults [149,198]. In the current study no improvements were seen for sarcopenia status of the post-hospitalized older adults. This might have been influenced by the low sample size in this study, the chosen criteria for sarcopenia assessment (EWGSOP2 algorithm based on handgrip strength (kg) and appendicular skeletal muscle mass (kg)) [38], and thereby the low number of

participants diagnosed with this condition compared with other screening criteria [199,200], as well as by the multifactorial nature of sarcopenia [40].

In contrast, the resistance training program after an acute period of hospitalization positively influenced the frailty status of the older adults in the current study. Regarding leucine-enriched protein supplementation after each training session (2 sessions/week), no additional beneficial effects were seen for either of the conditions, no sarcopenia nor frailty. Nevertheless, this is in line with some studies that have also failed to see further benefits with protein supplementation [146,190,196]. It seems that nutritional interventions might play an important role for preventing and/or improving sarcopenia and frailty, but there is still no consensus on which might be the best approach [40,150]. For example, for sarcopenia a multi-ingredient approach to dietary supplementation has been suggested as the most effective strategy [142].

According to myokines there is still much more research needed to clarify their role in improving muscle mass and strength, and thereby physical function, in older adults as contradictory studies have been published regarding myostatin, follistatin and irisin [156,159,162–168]. Our study results are far from shedding light on this area, but we believe that the **Study 4** might have added some starting points that need to be proved in the future. To our surprise, myostatin did not show the expected negative association with muscle mass related parameters in the current study (appendicular skeletal muscle mass (kg) and fat free mass index (kg/m^2)). This myokine has been often defined as a negative regulator of myogenesis [154], and our results, in accordance with other authors [162,166,201], suggest that maybe the role of myostatin is much more complex than just being a negative regulator. The small sample size in the current study did not permit to arise conclusive statements regarding myostatin, but neither for follistatin and irisin.

Overall, this study underscored, in accordance with the Study 3, the importance of implementing resistance training programs immediately post-discharge in older adults. Our results showed that with 12 weeks of resistance training we start to observe beneficial effects on physical function in this older adults' population. Thus, we suggested that, if prolonged, sarcopenia and frailty status will improve accordingly.

5. REFERENCES

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6. CONCLUSIONS AND CLINICAL HEALTH IMPLICATIONS

6.1 Conclusions

The conclusions of the current International Doctoral thesis are the following:

I) A high percentage of the hospitalized older adults at the internal medicine service of the Araba University Hospital in Vitoria-Gasteiz were at risk of malnutrition or malnourished, and showed an impaired physical function compared to their healthy counterparts. This decline within different physical tests was associated with worse nutritional status.

II) Handgrip strength and the SPPB, as well as its subtests, might help to complement the usual nutritional screening in hospitalized older adults. Hence, it seems that when physical function assessment is not feasible, nutritional status assessed by the MNA-SF might help to predict poor physical function in this population.

III) Malnutrition and frailty increased the risk to be classified as at severe comorbidity according to the Charlson Comorbidity Index, whereas being unfit for handgrip strength did not increase the risk. However, it seems that frailty might be a major contributor to the Charlson Comorbidity Index increase than nutritional status, as older inpatients classified as non-frail had lower values of Charlson Comorbidity Index regardless of their nutritional status. Nevertheless, the results of the current International Doctoral Thesis suggest that the use of the MNA-SF and the SPPB in geriatric hospital patients might help to predict poor clinical outcomes.

IV) Resistance training should be considered first-line strategy to maintain muscle mass and increase gains in physical function parameters immediately after discharge in older adults. Specifically, 12 weeks of supervised resistance training with one-hour session over two days/week seems enough to enhance strength and physical function variables in this population. No additional beneficial effects are seeing with leucine-enriched protein supplementation post-exercise, but its potential cannot be discarded. Further studies are needed regarding protein supplementation in post-hospitalized older adults.

V) Resistance training should be considered a primary countermeasure to combat and/or prevent sarcopenia and frailty in post-hospitalized older adults. However, the additional effects of an enriched-protein supplementation with resistance training to combat these conditions needs to be further studied. Findings regarding myokines are still contradictory, and the result from the current International Doctoral Thesis should be taken with caution. To contrast our results, future studies are needed with larger sample sizes to understand how myostatin responds to

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training stimuli at the cellular level as well as at systemic level and if these responses correspond with the training outcomes observed in different contexts.

6.2 Clinical health implications

The findings from the current International Doctoral Thesis might have clinical implications for the management of risk of malnutrition and/or poor physical function in hospitalized older adults. Hence, these findings have shown the association between the most widely and easy-to-use tools to screen malnutrition (MNA-SF) as well as poor physical function (handgrip strength and SPPB) in older inpatients, and the association of these tools with comorbidity risk in hospitalized older adults. Likewise, it could be suggested the use of either of the studied tools, depending on the clinical setting and/or older adults' characteristics, for a first step screening. This might be highly relevant as health-care professionals often have to cope with time-, resource- and space-related limitations in their daily clinical routine and consequently older inpatients at risk of malnutrition or malnourished and/or with poor physical function are often not identified. Hence, these conditions increase the health-economic burden as are usually related to negative clinical outcomes worsening the prognostic of hospitalization in older adults.

Lifestyle interventions based on nutritional support and resistance training are being recommended as the firs-line strategies to combat muscle mass and strength loss, and thereby sarcopenia and frailty. The design and implementation of interventions in line with those recommendations immediately post-discharge seems of great importance to recover from the deleterious effects of hospitalization and improve the clinical outcomes in older adults. In this regard, the current International Doctoral Thesis might have increased relevance as it proves that 12 weeks of resistance training (2 sessions/week) program immediately after discharge is an effective countermeasure to improve physical function in older adults. Although, the findings failed to show the additional beneficial effects of a leucine-enriched supplementation after each training session in the current International Doctoral Thesis, it does not totally discard the possible benefits of protein supplementation in different clinical population and/or even with a different supplementation protocol. Indeed, there are still many questions that need to be answered before a definitive statement is made regarding protein supplementation in older adults.

Overall, including screening tools to prevent and/or manage malnutrition as well as poor physical function into the daily clinical routine of hospitals seems of great importance. Even more in view of the current COVID-19 pandemic, which have further highlighted the vulnerability of older adults. Hence, the screening will help to design the most effective interventional strategy combining nutritional support and resistance training immediately post-discharge so as to ease the recovery of older adults. Finally, it is worth mentioning the

importance of including nutritionists as well as exercise specialists in clinical settings for an adequate and effective application of these proposed strategies.

7. ANNEX

7.1 Study 1: Nutritional Status and Physical Performance Using Handgrip and SPPB Tests in Hospitalized Older Adults.

Nutritional status and physical performance using handgrip and SPPB tests in hospitalized older adults

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Abbreviations:

SPPB: Short Physical Performance Battery

MNA-SF: Mini Nutritional Assessment-Short Form

CVD: Cardiovascular Disease

COPD: Chronic Obstructive Pulmonary Disease

Abstract

Background & Aims: Malnutrition and poor physical performance are highly prevalent within hospitalized older adults, and both have in common the loss of muscle mass. Likewise, there is growing interest in identifying markers of physical performance, other than just measuring muscle mass, that might be useful for managing malnutrition. This study aimed to (i) characterize the physical condition of hospitalized older adults in comparison to previously published reference percentile values of same age adults and (ii) to examine the association between the nutritional status and physical performance of older inpatients.

Methods: A total of 604 inpatients (age 84.3±6.8 years, 50.3% women) participated in this cross-sectional study. Patients were assessed for nutritional status (Mini Nutritional Assessment-Short Form (MNA-SF)) and physical performance (handgrip strength and the Short Physical Performance Battery (SPPB)).

Results: During hospitalization, 65.7% of the inpatients were at risk of malnutrition or malnourished. More than a half of the older inpatients were unfit (≤ P25) for handgrip strength (52.0%) and SPPB total score (86.3%) as well as for two of its subtests, gait speed (86.7%) and 5 times sit-to-stand (91.1%) tests. Patients' nutritional status was significantly associated with better physical performance within all tests (all $p < 0.001$), as their nutritional status improved so did their physical performance (all p for trend < 0.001). Hence, being at risk of malnutrition or malnourished significantly increased the likelihood for being classified as unfit according to handgrip strength (OR: 1.466, 95% CI: 1.045-2.056), SPPB total score (OR: 2.553, 95% CI: 1.592-4.094) and 4-m walking test (OR: 4.049, 95% CI: 2.469-6.640) (all $p < 0.05$), and as frail (OR: 4.675, 95% CI: 2.812-7.772) according to the SPPB frailty threshold ($p < 0.001$).

Conclusions: this study reinforces the use of handgrip strength and SPPB, as well as its subtests (gait speed and 5 times sit-to-stand tests), in hospitalized older adults as alternative measures of muscle mass for malnutrition management. Hence, it seems that risk of malnutrition or malnutrition assessed by MNA-SF might help to predict poor physical performance in older inpatients.

Key words: older adults, inpatient, malnutrition, handgrip, physical performance, muscle strength.

Introduction

Malnutrition is highly prevalent in older adults with greater numbers in hospitalized patients as well as in nursing homes [1]. Malnutrition occurs along with the aging process *per se* as well as with a background of chronic co-morbidities and/or acute conditions [2]. Malnourished older adults are at higher risk of fracture [3] and mortality [4, 5], and the recovery from any disease, trauma and/or surgery intervention is delayed [5]. In addition, malnutrition in older adults is associated with longer stays in hospital and higher readmissions rates with the subsequent economic burden for health care systems [4].

One of the most critical outcomes of malnutrition is the loss of muscle mass [6], which is exacerbated within hospitalized older patients due to inactivity [7] and the associated acute and/or chronic conditions [8]. Most of the techniques to measure muscle mass are not always available due to their high cost and/or time-consuming, or because they are not easy-to-use by any health-care professional nor practical within the daily hospital routine [6]. However, physical performance tests might be available even at the most resource-limited settings and are easy-to-use tools by any health-care professional [6]. Handgrip strength has been proposed as an alternative tool to estimate muscle mass as probably is one of the most affordable and easy-to-use tools in clinical settings due to its simple assessment and easy adaptation to almost every inpatient (bedridden or not) [6,9,10]. Thus, the identification of such surrogate measurements of muscle mass seems crucial for malnutrition management [6]. Likewise, the Short Physical Performance Battery (SPPB) has also gained attention and might be valuable, as it is widely used in clinical settings for physical performance assessment within older adult population [11].

Handgrip strength has been proposed as a biomarker for health status in older adults due to its clinical and prognostic value [12] and poor performance within the SPPB has been linked to all-cause mortality [13]. Reference values for the SPPB [14] and handgrip strength [15, 16] have been published describing older adults' population as well as general normative data by age groups for the SPPB [17] and handgrip strength [18]. Likewise, it is of clinical and public health interest to feature the physical status of the older inpatients in comparison to their healthy counterparts [10, 12]. This would add further clinical information to track the overall health status of patients and to design intervention programs in order to maintain and/or improve muscle mass and strength [15].

The usefulness of handgrip strength for nutrition assessment merits further research as there is conflicting data due to small sample sizes regarding older adults [19]. Similarly, the SPPB has shown a strong association with malnutrition in older inpatients [20], but the associations

of each of the subtests with malnutrition have not been studied yet in hospitalized older adults. This might be of interest as two of those subtests (gait speed and 5 times sit-to-stand tests) reflect muscle power, which has been suggested to be a better discriminatory predictor of functional performance than muscle strength [21].

Thereby, this study aimed to (i) characterize the physical condition of hospitalized older patients according to recently published reference percentile values for handgrip strength and for the SPPB total score and two of its subtests, and (ii) to examine the association between malnutrition and the physical performance of the studied sample.

Materials and Methods

Study design

This study was a cross-sectional secondary analysis conducted as part of the recruitment for a randomized controlled trial (ClinicalTrials.gov ID: NCT03815201) at the internal medicine service of the Araba University Hospital in Vitoria-Gasteiz (Spain) from September 2017 to July 2018. The study was approved by the Clinical Research Ethics Committee of the Araba University Hospital (CEIC-HUA: 2017-021) and complied with the revised ethical guidelines of the Declaration of Helsinki (revision of 2013). All patients were informed about the details of the research and signed an informed consent for their evaluation during hospitalization.

Participants

Members of the research team, with a wide experience in clinical settings, revisited the daily list of patients admitted to the internal service in order to assess eligibility. The reasons for hospitalization can be found in a study published by our research group [22] and coincide with those that can be expected for a geriatric population. The average length of hospital stay for older adults admitted to this hospital ward was 7.9 ± 5.2 days. Patients meeting the following criteria were eligible for inclusion and were evaluated within the first 3 days upon admission: ≥ 70 years old, ≥ 20 cut-off at the Mini Mental State Questionnaire, were able to walk alone or using assistive devices (cane, crutch,…), were able to understand and follow the instructions, and signed the informed consent. However, they were not eligible for evaluation if they had any of the following exclusion criteria: been suffering from severe dementia or Parkinson, been unable to stand and/or walk a short distance, been in critical medical condition or death, and if they had suffered any fracture of the upper or lower limbs in the last 3 months. For the current study, we included participants with valid data on nutritional status assessed by the Mini

Nutritional Assessment-Short Form (MNA-SF) score and physical performance evaluated using the handgrip strength and/or the SPPB.

From the 1878 hospitalized patients, a total of 775 (41.3%) patients met the inclusion criteria (Figure 1). However, 32 (4.1%) refused to be evaluated, 21 (2.7%) were moved to another medical service or hospital, and 113 (14.6%) had been discharged with not chance to be interviewed. Finally, 604 participants were included in the current study (n=5 did not have MNA-SF score data) (**Figure 1**).
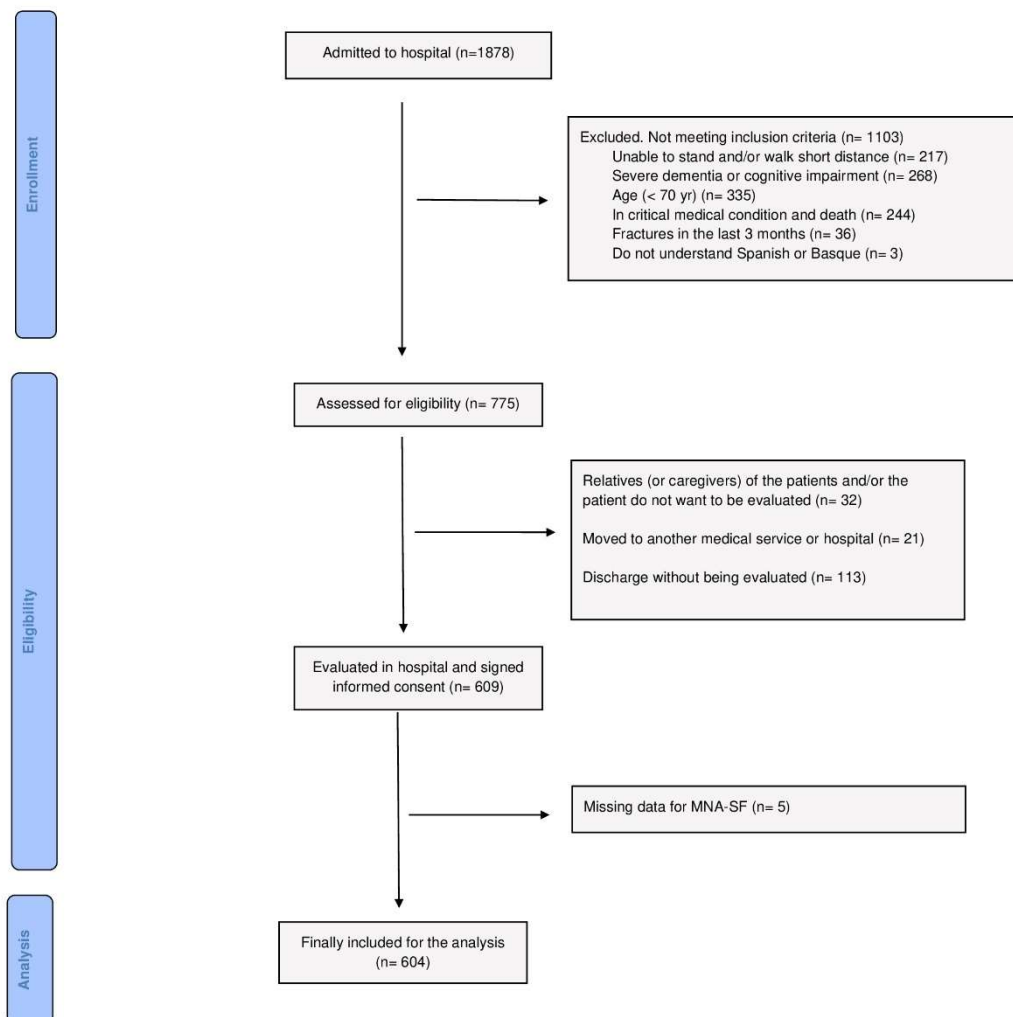

Fig. 1. Flow diagram of participants

Data collection

The medical history and number of drugs given to the patients at the time admitted to hospital were obtained by revising the clinical records. For the current study, polypharmacy was defined as the routine use of ≥ 5 drugs [23] and the comorbidity burden was defined by the Charlson Comorbidity Index [24].

Nutritional Assessment

Nutritional status was assessed by the MNA-SF (Nestlé Nutrition Institute, [25]) questionnaire directly with the patients and/or their respective relatives or caregivers. The MNA-SF has been proposed as a valid screening test to identify old institutionalized participants with malnutrition [25]. This questionnaire comprises 6-items and each answer has a numerical value contributing to the final score [25]. A maximum of 14 points can be obtained and depending on the score, the following categories are described: 0-7 points malnutrition, 8-11 points at risk of malnutrition and 12-14 points normal nutritional status [25]. The last item of the MNA-SF can be answered by body mass index estimation or by measuring the calf circumference [25]. As we had difficulties to measure height in several patients, we decided to use calf circumference following the standard protocol recommended by the International Society for the Advancement of Kinanthropometry. When possible, body mass (kg) was measured barefoot based on standardized protocols (OMROM HN-288, Digital Personal Scale, Barcelona, Spain).

For several analyses, the three MNA-SF categories were re-coded into two categories. Those at risk of malnutrition or with malnutrition were grouped together into "malnutrition or risk of malnutrition" as both are considered risk factors within older adult population, and the remaining category was "normal nutritional status".

Physical Performance Assessment

Physical performance was assessed by two tests: handgrip strength and the SPPB. Hence, the gait speed and the 5 times sit-to-stand tests, which are part of the SPPB, were also analysed separately to assess physical performance.

Dominant handgrip strength (kg) was measured by a handheld dynamometer (JAMAR® PLUS + Hand dynamometer) in a seating position, as it has been proposed for older adults in clinical practice [26]. Patients were classified as fit (>P25) or unfit (≤P25) according to reference percentile values for handgrip strength published by Dodds et al. [18].

Physical performance of the lower limbs was evaluated using the SPPB clinical tool [27]. The SPPB assessment methodology has been published elsewhere [27] and includes 3 subtests: 1) the standing balance test, 2) the gait speed test and 3) the 5 times sit-to-stand test. The total SPPB score ranges from 0 to 12, with the score of each subtest ranging from 0 to 4 points. According to the total score obtained, 4 clinically relevant categories have been defined for the SPPB: from 0 to 3, from 4 to 6, from 7 to 9 and from 10 to12 points [27]. It has been shown that scores below 10 points are associated with mobility-related disability [28] and/or with increased risk of death [13]. Hence, the SPPB has been proposed as a good discriminatory tool for frailty and the threshold for its assessment have been established at scores ≤ 9 [29, 30]. So, it was decided to classify those inpatients with scores ranging from 0 to 9 as "frail" and those with scores ranging from 10 to 12 as "non-frail" [13, 29, 30].

Patients were also classified independently as fit (>P25) or unfit (≤P25) for the SPPB total score, the gait speed test and the 5 times sit-to-stand test according to the reference percentile values published by Bergland et al. [17].

Statistical analysis

The distribution of the variables was verified using the Shapiro–Wilks test, skewness and kurtosis values and those variables with non-normal distribution were logarithmically transformed (i.e., age, body mass (kg), Charlson Comorbidity Index, SPPB total score as well as gait speed (m/s), 5 times sit-to-stand tests (sec), and MNA-SF score). Differences in sociodemographic and clinical characteristics between women and men were analysed using the independent Student *t* test and the Chi-square test for continuous and categorical variables, respectively. Univariable analysis was conducted to describe the distribution of the sample (absolute and relative frequencies) across the reference percentile values for handgrip strength, the SPPB total score, the gait speed test and the 5 times sit-to-stand test.

Pearson's correlation was used to analyse the association between continuous variables (the MNA-SF score with each physical performance test). Analysis of variance (polynomial) was done to compare the mean values for each physical performance test among the 3 nutritional status categories (normal nutritional status, risk of malnutrition and malnutrition) with Bonferroni adjustment. Binary logistic regression models were carried out to analyse the risk for being classified as unfit (within the different physical performance tests: handgrip strength, SPPB total score, gait speed test and 5 times sit-to-stand test) or frail for the SPPB total score according to the nutritional status ("malnutrition or risk of malnutrition" *vs*. "normal nutritional status").

All statistical analyses were done using the statistical software SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) with a level of significance of α = 0.05. Data are expressed as means ± SEM, unless other is indicated.

Results

Clinical characteristics of participants

Table 1 shows clinical characteristics of participants by gender. Women were older and had lower body mass than men (all $p < 0.01$, Table 1). It was also observed that women had significantly higher rates of depression, but lower rates of cardiovascular disease, chronic obstructive pulmonary disease, diabetes, kidney disease and neoplasia than men (all $p < 0.05$, Table 1). Hence, women had significantly lower Charlson Comorbidity Index score than men ($p < 0.001$, Table 1).

Men scored significantly higher in the MNA-SF test than women ($p < 0.005$, Table 1) and performed significantly better than women within all physical tests (all $p < 0.05$, Table 1).

Table 1 Characteristics of participants in the study by gender

	N	Total	N	Women	N	Men	P*
Age (years)	604	84.3 (6.8)	304	85.1 (6.9)	300	83.4 (6.5)	< 0.005†
Body mass (kg)[a]	589	67.2 (13.3)	296	62.8 (12.8)	293	71.6 (12.2)	< 0.001†
Number of drugs	604	7.2 (3.7)	304	7.1 (3.6)	300	7.4 (3.9)	0.742†
Polypharmacy (N, %)	604	455, 75.3	304	226, 74.3	300	229, 76.3	0.571
Depression (N, %)	604	56, 9.3	304	40, 13.2	300	16, 5.3	< 0.005
Diseases							
Hypertension (N, %)	604	446, 73.8	304	224, 73.7	300	222, 74.0	0.930
CVD (N, %)	604	179, 29.6	304	71, 23.4	300	108, 36.0	< 0.005
COPD (N, %)	604	121, 20.0	304	38, 12.5	300	83, 27.7	< 0.001
Diabetes (N, %)	604	206, 34.1	304	88, 28.9	300	118, 39.3	< 0.05
Kidney disease (N, %)	604	109, 18.0	304	42, 13.8	300	67, 22.3	< 0.05
Hepatic disease (N, %)	604	13, 2.2	304	6, 2.0	300	7, 2.3	0.761
Neoplasia (N, %)	604	124, 20.5	304	40, 13.2	300	84, 28.0	< 0.001
Dementia (N, %)	604	23, 3.8	304	16, 5.3	300	7, 2.3	0.060
Parkinson (N, %)	604	19, 3.1	304	10, 3.3	300	9, 3.0	0.839
Charlson Comorbidity Index[b]	597	6.3 (2.1)	300	5.8 (1.7)	297	6.7 (2.2)	< 0.001†
Physical Function							
Handgrip (kg)[c]	603	19.6 (8.3)	303	14.6 (5.5)	300	24.5 (7.6)	< 0.001
SPPB total score[d]	598	5.4 (3.1)	300	4.7 (2.9)	298	6.1 (3.2)	< 0.001†
0-3 (N, %)	598	190, 31.8	300	119, 39.7	298	71, 23.8	< 0.001
4-6 (N, %)	598	193, 32.3	300	99, 33.0	298	94, 31.5	
7-9 (N, %)	598	138, 23.1	300	58, 19.3	298	80, 26.8	
10-12 (N, %)	598	77, 12.9	300	24, 8.0	298	53, 17.8	
Gait speed test (m/s) [d]	598	0.5 (0.3)	300	0.4 (0.2)	298	0.6 (0.3)	< 0.001†
5 times sit-to-stand test (sec)[e]	394	19.6 (8.7)	180	20.9 (9.8)	214	18.4 (7.5)	< 0.05†
Nutritional Status							
Mini Nutritional Assessment-Short Form score	604	10.0 (2.5)	304	9.6 (2.6)	300	10.4 (2.3)	< 0.005†
Normal nutritional status (N, %)	604	207, 34.3	304	87, 28.6	300	120, 40.0	0.001
At risk of malnutrition (N, %)	604	293, 48.5	304	151, 49.7	300	142, 47.3	
Malnourished (N, %)	604	104, 17.2	304	66, 21.7	300	38, 12.7	

Abbreviations: CVD: cardiovascular disease; COPD: chronic obstructive pulmonary disease; SPPB total score: Short Physical Performance Battery total score. Values are means and standard deviations unless otherwise is indicated. *p refers to differences between men and women analyzed by t test for independent samples in continuous variables and Chi-squared test for categorical variables. †means and standard deviations are presented for not transformed variables to ease interpretation, but p were obtained by t test for independent samples with logarithmically transformed continuous variables.

[a]Data were missing for 15 patients

[b]Data were missing for 7 patients

[c]Data was missing for 1 patient

[d]Data were missing for 6 patients

[e]Data were missing for 210 patients

Physical performance in hospitalized patients

Table 2 shows the distribution of the hospitalized patients according to reference percentile values [17, 18]. For handgrip strength test, almost 50% of women and more than one half (58.3%) of men patients were ≤ P25. However, more than 80% of patients were ≤ P25 for SPPB total score (88.7% women and 83.9% men), gait speed test (92.4% women and 81.2% men) and 5 times sit-to-stand test (91.1% women and 91.1% men).

Table 2 Population distribution according to percentiles for handgrip (kg), Short Physical Performance Battery (SPPB) total score, gait speed test (m/s) and 5 times sit-to-stand test (sec)

	≤P5	>P5 - ≤P10	>P10 - ≤P25	>P25 - ≤P50	>P50 - ≤P75	>P75 - ≤P90	>P90
Handgrip (kg) (N, %)	-	177, 29.4	136, 22.6	183, 30.3	65, 10.8	29, 4.8	13, 2.2
Women	-	80, 26.4	58, 19.1	99, 32.7	37, 12.2	21, 6.9	8, 2.6
Men	-	97, 32.3	78, 26.0	84, 28.0	28, 9.3	8, 2.7	5, 1.7
SPPB total score (N, %)	341, 57.0	86, 14.4	89, 14.9	57, 9.5	7, 1.2	-	18, 3.0
Women	170, 56.7	50, 16.7	46, 15.3	22, 7.3	7, 2.3	-	5, 1.7
Men	171, 57.4	36, 12.1	43, 14.4	35, 11.7	-	-	13, 4.4
Gait speed test (m/s) (N, %)	414, 69.2	42, 7.0	63, 10.5	48, 8.0	22, 3.7	6, 1.0	3, 0.5
Women	230, 76.7	20, 6.7	27, 9.0	15, 5.0	5, 1.7	3, 1.0	-
Men	184, 61.7	22, 7.4	36, 12.1	33, 11.1	17, 5.7	3, 1.0	3, 1.0
5 times sit-to-stand test (sec) (N, %)	235, 59.6	69, 17.5	55, 14.0	26, 6.6	7, 1.8	2, 0.5	-
Women	112, 62.2	33, 18.3	19, 10.6	12, 6.7	3, 1.7	1, 0.6	-
Men	123, 57.5	36, 16.8	36, 16.8	14, 6.5	4, 1.9	1, 0.5	-

Abbreviations: SPPB: Short Physical Performance Battery. Data are presented as number and %. Handgrip percentiles according to Dodds et al [18]; SPPB total score, gait speed test (m/s) and 5 times sit-to-stand test (sec) percentiles according to Bergland et al. [17].

Association of nutritional status and physical performance

The associations of nutritional status, assessed by the MNA-SF test, with the physical performance tests are shown in **Table 3**. Better nutritional status was significantly associated with better performance in handgrip strength, gait speed and the 5 times sit-to-stand tests, as well as with higher SPPB total score (all $p < 0.001$, Table 3).

Table 3 Association of nutritional status assessed by the Mini Nutritional Assessment-Short Form (MNA-SF) test with physical function tests

	Handgrip (kg)		SPPB (total score)		Gait speed test (m/sec)		5 times sit-to-stand test (sec)	
	r	P	r	P	r	P	r	P
MNA-SF (lineal)	0.286	< 0.001	0.315	< 0.001	0.266	< 0.001	-0.189	< 0.001

Abbreviations: MNA-SF: Mini Nutritional Assessment-Short Form; SPPB (total score): Short Physical Performance Battery (total score). Unadjusted correlation tests. Pearson's correlations were calculated with the logarithmicallytransformed variables, except for handgrip strength.

Likelihood of being classified as unfit or frail by malnutrition status

The likelihood for being classified as unfit or frail within different physical assessment tests according to the nutritional status are shown in **Figure 2**. It was observed that patients classified as being at risk of malnutrition or having malnutrition had higher likelihood of being classified as unfit for handgrip strength test ($p = 0.027$), SPPB total score ($p < 0.001$), and gait speed test ($p < 0.001$), as well as being frail according to the SPPB frailty threshold ($p < 0.001$) (Figure 2). Hence, patients at risk of malnutrition or having malnutrition had > 4.5 times higher risk for being classified as frail according to the SPPB frailty threshold, and 4.0 times for being classified as unfit for the gait speed test, > 2.5 times for the SPPB total score and 1.5 times for the handgrip strength test (Figure 2). However, being at risk of malnutrition or having malnutrition did not increase the likelihood of being classified as unfit for the 5 times sit-to-stand test ($p = 0.458$, Figure 2). Hence, when those older inpatients that were not able to perform the 5 times sit-to-stand test were included within the unfit group the results did not substantially change (OR: 1.889, 95% CI: 0.952-3.749, $p = 0.069$).
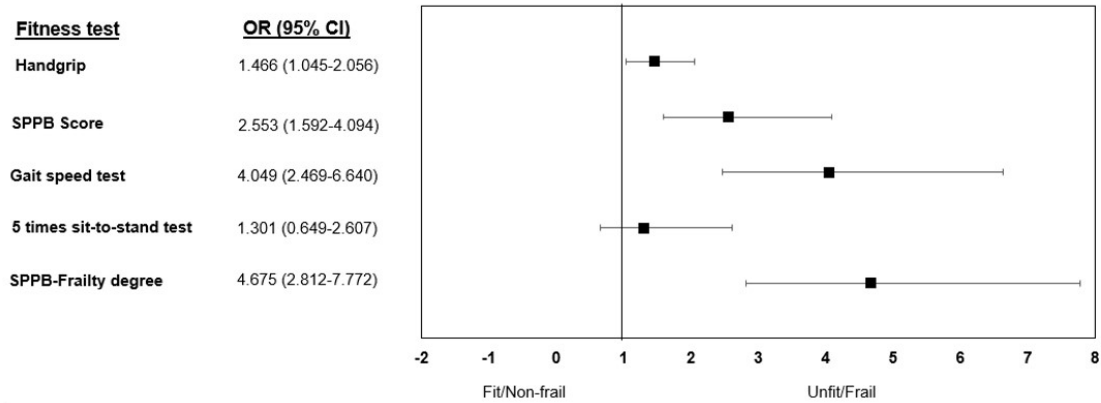
Fig. 2. Odd Ratios and 95% confidence intervals for being classified as unfit or frail according to different fitness tests (handgrip, SPPB, gait speed test and 5 times sit-to-stand test) in those patients at risk of malnutrition or with malnutrition (MNA-SF total score ≤ 11). Abbreviations: MNA-SF: Mini Nutritional Assessment-Short Form; Unfit-SPPB: Unfit-Short Physical Performance Battery; Frailty-SPPB: frailty-Short Physical Performance Battery. Unadjusted odds ratios. Handgrip unfit assessment according to Dodds et al. [18] percentiles; Short Physical Performance Battery unfit assessment according to Bergland et al. [17] percentiles; gait speed test unfit assessment according to Bergland et al. [17] percentiles; 5 times sit-to-stand test unfit assessment according to Bergland et al. [17] percentiles; Frailty assessment according to the Short Physical Performance Battery frailty threshold

Differences within each physical performance test according to the nutritional status categories are shown in **Figure 3**. It was observed that as the nutritional status worsen the performance within all the physical tests declined too (all *p* for trend < 0.001, Figure 3).
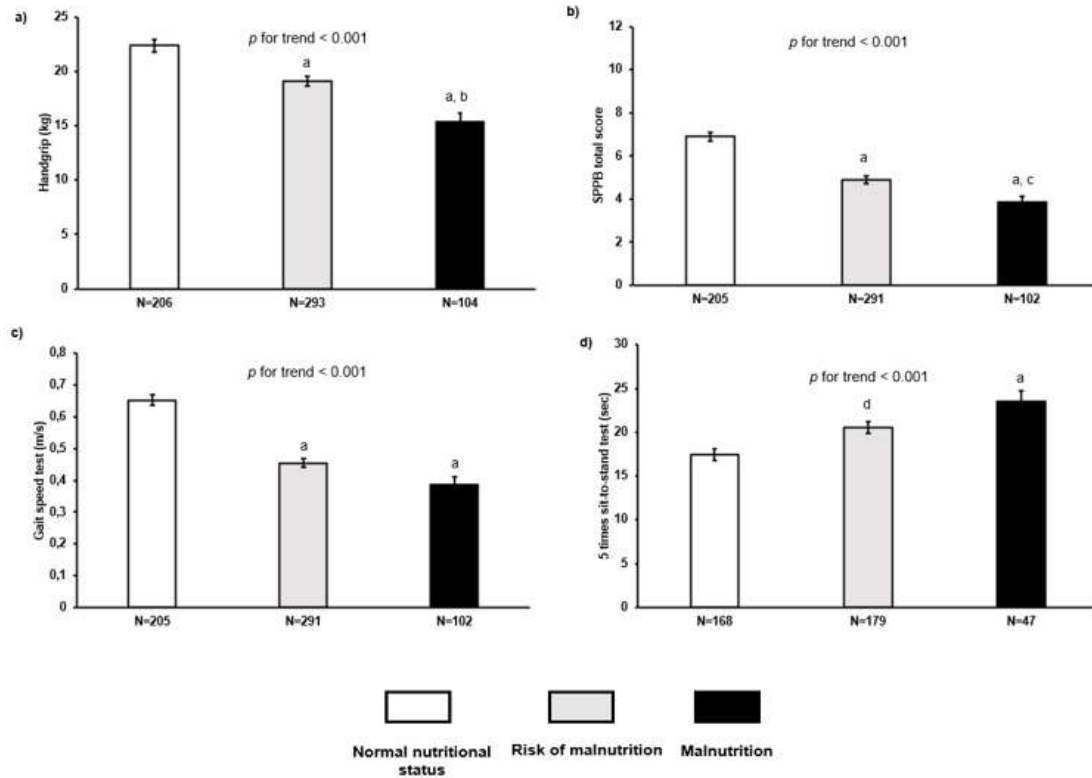
Fig. 3. Differences among physical function parameters according to the nutritional status categories. (a) handgrip (kg) (b) Short Physical Performance Battery (SPPB) total score (c) gait speed test (m/s) (d) 5 times sit-to-stand test (sec). Abbreviations: SPPB total score: Short Physical Performance Battery score. a: $p < 0.001$ denotes significant differences between patients with normal nutritional status and patients at risk of malnutrition or with malnutrition. b: $p < 0.001$ denotes significant difference between patients at risk of malnutrition and with malnutrition. c: $p < 0.05$ denotes significant difference between patients with malnutrition and patients at risk of malnutrition. d: $p < 0.005$ denotes significant difference between patients with normal nutritional status and patients at risk of malnutrition. Unadjusted analysis of variance (polynomial)

Discussion

The main findings of the current study were that hospitalized older patients (≥ 70 years old) showed an impaired physical performance compared to their healthy counterparts, and that this decline within different physical tests was associated with worse nutritional status. Hence, being at risk of malnutrition or malnourished increased the risk for being classified as unfit for the handgrip test, the SPPB score and the gait speed test, and as frail according to the SPPB frailty threshold. Thus, the early identification of those patients malnourished or at risk of malnutrition seems important as it might also help to identify those patients at risk of impaired physical performance and/or frailty in clinical settings.

Normative reference values help to track the physical performance of an individual over time in contrast to their healthy counterparts [15] and might add clinical value to the physical screening of hospitalized older adults. In the current study, more than a half of the older inpatients were ≤P25 for handgrip strength [18], and almost all measured patients were below ≤P25 for the SPPB score, the gait speed test, and the 5 times sit-to-stand test [17], being more than 50% of them below P5. Thus, the older inpatients in this study exhibited a poor physical performance which fell far below what it would have been considered appropriate for their age. However, when our results are compared to those from hospitalized older adults with a mean age of ≥ 80 years old [21, 31, 32], patients in our study showed better performance within handgrip strength, SPPB total score and 5 times sit-to-stand test. These results suggest that the hospitalized older adults in our study were in better physical condition, although sample sizes in those studies were smaller. Nevertheless, when our results are compared to other studies with larger sample sizes and a mean age around 68 years old, the older inpatients in our study showed worse physical performance, especially male patients [33, 34]. Indeed, confirming that muscle strength and function decrease with aging and the decrease seems steeper within men [15, 35].

Our study also showed that the 65.7% of the hospitalized older adults were at risk of malnutrition or malnourished assessed by the MNA-SF questionnaire during their hospitalization. This prevalence is within the range showed by other studies carried in clinical settings [20,36–40], confirming that a high proportion of older inpatients are already malnourished or that are at high risk of malnutrition [41].

It is well-known that a consequence of malnutrition is muscle mass loss, with the subsequent negative effects on muscle function and, therefore, on physical performance [6]. This could be reflected by the results from this study, showing associations between the nutritional status and handgrip strength and SPPB score as well as its subtests. Hence, according

to those physical performance tests, significant differences were seen between patients at risk of malnutrition or malnourished and with a normal nutritional status. To our knowledge, this is the first study examining the association of both handgrip strength and SPPB, as well as two of its subtests separately, with nutritional status in a large sample of hospitalized older adults. Recently, Ramsey et al. [42] published a similar study, but in geriatric outpatients, confirming a positive association between nutritional status and SPPB score, gait speed, and 5 times sit-to-stand tests. However, contrary to our results, but in accordance with other studies of hospitalized older adults [19,43,44], Ramsey et al. [42] did not observe any association between handgrip strength and nutritional status. Although, handgrip strength has been proposed as a potentially useful and rapid tool for nutritional assessment in hospital patients [9,34,45,46], this needs further research in older adult inpatients due to conflicting data. Those studies reporting an association between handgrip strength and nutritional status, included patients older than 18 years old encompassing a wide age range [9,34,45,46], whereas in studies where no association have been seen age was limited to ≥65 years old [19,43,44]. These study characteristics and the smaller sample sizes accompanied by the different nutritional screening tool employed in those studies [19,43,44], might have hidden any association limiting comparisons with our study.

The usage of the SPPB and its subtests for nutritional assessment has not been yet studied [13], albeit an association between this battery and the nutritional status of older adults has been confirmed in several studies [20,42,47,48]. In agreement with our findings, Ramsey et al. [42] reported significant associations of the SPPB and its subtests with malnutrition assessed by the Short Nutritional Assessment Questionnaire in geriatric outpatients. Therefore, the SPPB as well as each of its subtests might have individual value in relation to malnutrition. Hence, Mendes et al. [49] suggested that gait speed might have a potential capacity to identify poor nutritional outcomes, as it showed high sensitivity values similar to those of nutritional assessment tools. In this line, we also observed that being at risk of malnutrition or malnourished increased more than twice or four times the risk for been classified as unfit according to the SPPB total score and the gait speed test, respectively, in comparison to handgrip strength, which increased the risk 1.5 times. These results suggest that upper and lower limbs' strength might be affected differently by nutritional status [42], as it occurs with the ageing process [50]. However, the same significant results were not shown for the 5 times sit-to-stand test. This test is suggested to capture advanced stages of disability, but it has some limitations as older adults that are not able to perform the test have no time recorded for it [11,27]. The proposed normative values for the 5 times sit-to-stand test by Bergland et al. [17] were based

on the time needed to perform the test. So, those older inpatients unable to perform the test were not included within the fit/unfit classification. Thus, this inability to perform the test by a high percentage of the older adults in the current study along with the low number of patients classified as fit (only 35 patients) might have limited the results.

Finally, a relevant finding of our study was that those patients at risk of malnutrition or malnourished had >4.5 higher risk of being classified as frail according to the SPPB frailty threshold. This association between malnutrition and frailty has been reported by other studies, but frailty assessment was based on different criteria other than SPPB categories, such as the Frailty Instrument for Primary Care of the Survey of Health, Ageing and Retirement in Europe [51] and the Fried frailty criteria [52]. Indeed, those frailty criteria contained items that were shared by the MNA-SF questionnaire, so it is not surprising to have found an association [51,52]. However, this was not the case in our study as frailty assessment was based only on physical performance measurement (SPPB frailty threshold), without subjective questions shared by the MNA-SF. Thus, our study reinforces the link between malnutrition and physical performance level [52].

Physical performance assessment by the SPPB might have some limitations in clinical settings. Indeed, the assessment by this battery requires some space and time to perform the different subtests as well as the adequacy to a standardized protocol [27]. Thus, the results from this study reinforce the use of the MNA-SF for those clinical settings where is not feasible to assess physical performance by the SPPB. Nevertheless, once the SPPB is included into the daily routine of clinical settings, its assessment might not take more time than other tests [29] and given its ability to predict adverse health outcomes its assessment could be preferred in certain clinical settings [13].

In conclusion, our study brings new insights into malnutrition management, reinforcing the use of handgrip and SPPB, as well as its subtests, in hospitalized older adults to complement nutritional screening [6]. In addition, it seems that when physical performance assessment is not feasible, nutritional status assessed by the MNA-SF might help to predict poor physical performance in hospitalized older adults.

Strengths and limitations

One of the strengths of the study is that to the best of our knowledge this is the first study including a large sample (n=604) of hospitalized older adults (≥70 years old) to examine their nutritional status and its association with the most widely and easy-to-use physical performance tests in clinical settings (handgrip strength and SPPB). However, the reference

percentile values we used in our study were from European countries other than Spain. Thus, it would be interesting for future studies to publish standardized reference values for the Spanish population. Another limitation of our study is its cross-sectional design that limits to determine any causality. In addition, our results cannot be extrapolated to older inpatients not meeting our inclusion criteria and to other clinical settings, such as nursing homes and/or to community-dwelling older adults. Future studies in different context should examine the usefulness of the different physical performance tests to complement the nutritional assessment.

Acknowledgments

We would like to acknowledge all participants and their families for allowing us to conduct the nutritional and physical assessments during their hospitalization. We would also like to thank all professionals and pre/postgraduate students who have been involved in data collection and measurements as well as the Araba University Hospital in Vitoria-Gasteiz, and the professionals working there, for providing their facilities and enabling our visits to the hospitalized patients.

Statement of Authorship

IL, AB, and JI designed the study, MA and MU collected the data, MA, MM and IL interpreted the data and drafted the manuscript, MA, AB, MM, MU, ARL, IT, JB, JI, and IL have approved the submitted version and agree to be personally accountable for the author´s own contributions and for ensuring that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and documented in the literature.

Conflict of Interest Statement

Maria Amasene, Ariadna Besga, María Medrano, Miriam Urquiza, Ana Rodriguez-Larrad, Ignacio Tobalina, Julia Barroso, Jon Irazusta and Idoia Labayen declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Funding

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7.2 Study 2: Malnutrition and Poor Physical Function are Associated with Higher Comorbidity Index in Hospitalized Older Adults

Malnutrition and Poor Physical Function are Associated with Higher Comorbidity Index in Hospitalized Older Adults

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Keywords: chronic diseases, geriatrics, inpatients, nutritional status, muscle strength, mortality.

Abstract

Background: Charlson Comorbidity Index (CCI) is the most widely used method to measure comorbidity and predict mortality. There is no evidence whether malnutrition and/or poor physical function are associated with higher CCI in hospitalized patients. Therefore, this study aimed to (i) analyze the association between the CCI with nutritional status and with physical function of hospitalized older adults, and (ii) to examine the individual and combined associations of nutritional status and physical function of older inpatients with comorbidity risk.

Methods: A total of 597 hospitalized older adults (84.3±6.8 years, 50.3% women) were assessed for CCI, nutritional status (the Mini Nutritional Assessment-Short Form, MNA-SF) and physical function (handgrip strength and the Short Physical Performance Battery).

Results: Better nutritional status ($p < 0.05$) and performance within handgrip strength and the SPPB were significantly associated with lower CCI score among both males ($p < 0.005$) and females ($p < 0.001$). Patients with malnutrition or risk of malnutrition (OR: 2.165, 95% CI: 1.408-3.331, $p < 0.001$) as well as frailty (OR: 3.918, 95% CI: 2.326-6.600, $p < 0.001$) had significantly increased risk for being at severe risk of comorbidity. Patients at risk of malnutrition or malnourished had higher CCI score regardless of being fit or unfit according to handgrip strength (p for trend<0.05) and patients classified as frail had higher CCI despite their nutritional status (p for trend<0.001).

Conclusions: The current study reinforces the use of the MNA-SF and the SPPB in geriatric hospital patients as they might help to predict poor clinical outcomes, and indirectly post-discharge mortality risk.

Introduction

The prevalence of chronic diseases has substantially increased in the last years along with the aging of the population [1,2]. It has been reported that in Europe, 50% of older adults have ≥ 2 chronic diseases [3].

Hospitalization rates increase linearly with the number of chronic diseases [4] and thereby healthcare costs [5]. In clinical settings, the Charlson Comorbidity Index (CCI) is the most widely used method to measure comorbidity and predict mortality [6]. This index considers the number and severity of the concurrent diseases with the aim of identifying those patients at risk for negative health outcomes [4].

Malnutrition is often observed among older adults at hospital admission [7], hindering recovery from diseases, surgery or trauma, worsening the prognostic [8,9] and increasing healthcare costs [5]. Hence, being malnourished has been associated with higher risk of in-hospital mortality [9,10] as well as with higher mortality in the short- [11–14] and long-term after discharge [10,14,15].

Several performance-based physical tests have shown good validity for predicting poor health outcomes [16,17]. Handgrip strength has been proposed as an important biomarker of health status [18], and a potential predictor of comorbidity [19,20] and mortality [21–23]. Similarly, the Short Physical Performance Battery (SPPB) seems to be able to predict disability [24] and mortality risk [25,26].

Thereby, although it is not new that malnutrition and markers of physical function contribute to increase comorbidity [18,27] and lastly mortality [25,27,28], there are few studies aiming to evaluate the associations of comorbidity assessed by CCI with malnutrition and poor physical function [13,29,30]. Hence, there are no previous studies examining whether malnutrition and poor physical function independently or in combination, are associated with higher CCI in hospitalized patients. Therefore, this study aimed to (i) analyze the association between the CCI, with nutritional status as well as with physical function of hospitalized older adults, and (ii) to examine the individual and combined associations of nutritional status and physical function of older inpatients with comorbidity risk.

Methods

Study design

This cross-sectional study is a secondary analysis, with the CCI variable as the endpoint of the study. This study was based on the data obtained during the recruitment for a randomized controlled trial (ClinicalTrials.gov ID: NCT03815201), which was conducted in Vitoria-Gasteiz (North of Spain) at the internal medicine service of the Araba University Hospital from September 2017 to August 2018. The study was approved by the Clinical Research Ethics Committee of the Araba University Hospital (CEIC-HUA: 2017-021) and is in line with the revised ethical guidelines of the Declaration of Helsinki (revision of 2013). All patients were informed about the details of the research and signed an informed consent before participating in the study.

Participants

The daily list of hospitalized patients at the internal medicine service was revisited to assess whether patients were eligible for evaluation or not by members of the research team with a wide experience in clinical settings. Patients were eligible for evaluation if they meet the following inclusion criteria: ≥ 70 years old, a punctuation of ≥ 20 at the Mini Mental State Questionnaire (MMSE), were able to walk alone or using a walking stick or walking frame, were able to understand and follow the instructions. However, they were not eligible for evaluation if they had any of the following exclusion criteria: been suffering from severe dementia or Parkinson, been unable to stand and/or walk a short distance, been in critical medical condition (e.g., need of palliative care and/or advance cancer) or death, and if they had suffered any fracture of the upper or lower limbs in the last 3 months. Hence, patients with no valid data regarding nutritional status assessed by the Mini Nutritional Assessment-Short Form (MNA-SF), the CCI and physical function (no data for handgrip strength nor SPPB), were excluded for analysis in this study.

Throughout the study duration 1878 patients were admitted to the internal medicine service, from them 1103 (58.7%) inpatients did not meet the inclusion criteria, whereas 775 (41.3%) were eligible for evaluation, and, finally, 597 patients (98.0% of the eligible patients) were finally included in the analyses. The flowchart of the study and reasons for the participant exclusion are detailed in **Figure 1**.

Data collection

Patients' clinical records were revisited to assess their medical history and number of drugs given to the patients at the admission to the hospital. Polypharmacy was considered as the routine use of ≥ 5 drugs [31].

Comorbidity risk

Comorbidity burden was defined according to the CCI [32]. The estimation of this index is based on age (divided into 5 ranges) and 17 different categories of comorbidity [32]. Each age range and category have an associated score (from 1 to 6, the latter based on the severity of the condition), and then all are summed contributing to the total score [32]. Thereafter, 3 different categories are defined to classify comorbidity risk within patients: 1) 1-2 points mild risk, 2) 3-4 points moderate risk, and 3) ≥ 5 points severe risk [32]. In the current study, all the participants were ≥ 70 years old, thereby we did not have any patients scoring 1-2 points due to the age-adjusted scoring [32].

Nutritional assessment

The MNA-SF questionnaire was used to assess patients' nutritional status by directly asking the patients and/or their caregivers. The MNA-SF is widely used in clinical settings and it has shown a high sensitivity [33,34]. For the current study those at risk of malnutrition and malnourished were grouped together into "malnutrition or risk of malnutrition" category, as both are considered risk factors within older adult population, and the remaining category was "normal nutritional status".

Physical function assessment

Two different tests were used to assess physical function: handgrip strength and the SPPB. Dominant handgrip strength (kg) was measured by a handheld dynamometer (JAMAR® PLUS + Hand dynamometer) in a seating position, as it has been proposed for older adults in clinical practice [35]. Those patients whose handgrip strength was ≤ P25 as compared with reference percentile values [36] were classified as unfit.

The SPPB clinical tool was chosen to measure physical function [37]. The SPPB consists of 3 subtests: 1) the standing balance test, 2) the gait speed test and 3) the 5 times sit-to-stand test. The total SPPB score ranges from 0 to 12, with higher scores reflecting better functional status, and it is divided into 4 categories: from 0 to 3, from 4 to 6, from 7 to 9 and from 10 to 12 points [37]. It has been proposed that scores ≤ 9 points might help to detect frail older adults

[38, 39]. Hence, it has also been shown that scores below 10 points are associated with increased risk of death [25]. Thus, for the current study, it was decided to classify scores ranging from 0 to 9 as "frail" and scores ranging from 10 to 12 as "non-frail" [25,38,39].

Statistical analysis

The Kolmogorov-Smirnov test was employed to verify the distribution of the variables, and those with non-normal distribution were logarithmically transformed (i.e., age, body mass (kg), MNA-SF score, handgrip strength (kg) and SPPB total score). Differences in sociodemographic and clinical characteristics between patients at moderate and severe comorbidity risk were analysed using the independent Student *t* test and the Chi-square test for continuous and categorical variables, respectively.

Linear regression analysis was used to examine the association between the dependent (CCI) and independent (performance in fitness tests and MNA-SF score) variables and data were tested for gender interaction. Analysis of variance (polynomial) was done to examine the synergetic association of nutritional status and performance within each physical test with CCI by Bonferroni adjustment. Binary logistic regression models were carried out to analyse the likelihood for being at severe comorbidity risk according to physical condition (unfit or frail *vs.* fit or non-frail) as well as to nutritional status (at risk of malnutrition or malnourished *vs.* normal nutritional status).

All statistical analyses were done using the statistical software SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) with a level of significance of α = 0.05. Data are expressed as means ± SEM.

Results

Table 1 shows the characteristics of participants by comorbidity risk categories. Briefly, those patients with severe risk of comorbidity were significantly older and had significantly higher rates of polypharmacy than those with moderate risk (all *p* < 0.001, Table 1).

Participants with severe risk of comorbidity scored significantly lower in the MNA-SF (*p* < 0.001) and performed significantly worse within handgrip and SPPB tests (all *p* < 0.005) than those patients with moderate comorbidity risk.

Association of comorbidity risk with nutritional status and physical function

Table 2 shows the associations of CCI score with the nutritional status and performance based physical tests. Higher punctuation in the MNA-SF (*p* < 0.05) as well as better performance

within handgrip strength ($p < 0.005$) and the SPPB ($p < 0.005$) were significantly associated with lower CCI score among males and females.

Likelihood for being at severe risk of comorbidity according to nutritional status and physical function

Figure 2 shows the likelihood for being at severe risk of comorbidity according to the nutritional status and the physical function. It was observed that the likelihood for being at severe risk of comorbidity was 2.0 times higher in patients at risk of malnutrition or malnourished (OR: 2.165, 95% CI: 1.408-3.331, $p < 0.001$) and 3.5 times higher in frail patients (OR: 3.918, 95% CI: 2.326-6.600, $p < 0.001$). In contrast, being unfit for handgrip strength did not increase the risk for being at severe risk of comorbidity (OR: 0.988, 95% CI: 0.646-1.512, $p = 0.956$).

Comorbidity risk according to the combination of the nutritional status and physical function of participants

Combined associations of nutritional status and performance-based categories on comorbidity risk are shown in **Figure 3**. It was observed that patients at risk of malnutrition or malnourished had higher CCI score regardless of being fit or unfit according to handgrip strength (p for trend < 0.05, panel A). Among fit patients, those with normal nutritional status had lower CCI score than those at risk of malnutrition or malnourished (5.7 *vs*.6.6, respectively, $p < 0.005$, panel A). Regarding the SPPB frailty threshold, patients classified as frail had higher CCI despite their nutritional status (p for trend < 0.001, panel B). Among those participants with normal nutrition status, those classified as frail had higher CCI score than non-frail patients (6.3 *vs*. 4.9, respectively, $p < 0.001$, panel B). Nevertheless, those frail patients at risk of malnutrition or malnourished had also higher CCI score in comparison to their no-frail counterparts with normal nutritional status (6.5 *vs*. 4.9, respectively, $p < 0.001$, panel B).

Discussion

The primary findings of the current study were that those hospitalized older adults aged ≥ 70 at risk of malnutrition or malnourished and frail according to the SPPB frailty threshold, had significantly higher risk of being at severe risk of comorbidity than their peers with normal nutritional status and non-frail (SPPB frailty threshold). Hence, older inpatients (≥ 70 years old) classified as non-frail had lower values of CCI regardless of their nutritional status. Thus, nutritional status and physical function assessment might help to predict indirectly mortality risk among older adult population in clinical settings.

Aging is considered an important risk factor for most of the diseases and conditions limiting healthspan [40]. Having used the CCI that includes an age-associated score according to age-ranges limits comparison with other studies [28–30] and makes difficult to account for the contribution of any disease to the CCI score within each inpatient. So, to observe the pattern of chronic diseases within the older inpatients in this study (≥ 70 years old), age was removed from the CCI scoring. Likewise, the 87.6% of the inpatients in this study showed at least one chronic disease, and from those the 74.8% scored ≥ 2 for the CCI showing more than one chronic disease and/or unless a severe chronic disease. From those 74.8% of the older inpatients, the 81.3% were ≥ 80 years old. These results are in line with a previous study conducted in Italy concluding that 86% of the adults older than 65 years lived with at least one chronic disease [2], and with other studies showing that multimorbidity increases with age [1,3]. Thus, these results reinforce aging as an important risk factor for increasing comorbidity burden. Hence, in the current study patients at severe risk of comorbidity were significantly older, had higher rates of polypharmacy and showed higher rates of chronic diseases as well as worse nutritional status and physical function compared to those at moderate risk of comorbidity.

Our results show that higher punctuation in the MNA-SF and performance-based physical tests were inversely associated with the CCI score among older inpatients. Previous studies aiming to measure the health-related consequences of malnutrition used different data other than CCI, such as length of hospital stay [41], readmission rates [12], morbidity of a specific disease [8] or short- [11,14] and long-term mortality [15]. There are only few studies using CCI as primary variable for that aim, and in contrast to our findings, they reported no association between nutritional status and comorbidity [29,30]. The different methodology used to assess nutritional assessment in those studies [29,30] limits comparison with the results obtained in this study. Nevertheless, as previously reported, the MNA-SF test performed well predicting unfavourable clinical outcomes [42] and is proposed to be the first choice for geriatric hospital patients [43]. Hence, this might be reflected by the twofold increase for severe comorbidity risk seen within malnourished older adults in the current study.

Similarly, handgrip strength has been proposed as a health biomarker [18]. Hence, previous studies showed an inverse association between handgrip strength and multimorbidity [19,20], which is in line with findings of the current study, as every increase in handgrip strength was negatively associated with CCI score. However, the current study failed to show an increased risk for severe comorbidity according to handgrip strength. This might be due to the handgrip percentiles used as reference, which were based on normative data [36], and/or due to the sex-interaction seen for handgrip strength, as the inverse association between handgrip

strength and CCI was stronger among older women in the current study. Similar findings were observed in the study of Volaklis et al. [19] where low handgrip strength was associated with an increased odds to be multimorbid among older women, but not men. Physiological mechanisms were suggested to explain sex-related differences for the relationship between handgrip strength and morbidity [19]. Nevertheless, although an increased risk for mortality was shown along with a declined in handgrip strength among older adults [21,22], it has also been suggested that the relation between muscle strength and mortality is not direct [21], and that behind that interaction there might be other factors underlying mortality [21], such as the number of diseases and/or their severity, which is accounted within the CCI scoring. Thus, this hypothesis might be reinforced by this study, although further research is needed to confirm it.

The SPPB has gained attention due to its ability to predict mortality risk [25,26] and its association with frailty [38]. Frailty has been linked to multimorbidity in several studies as shown by a recent systematic review and meta-analysis [44], but none of those studies used the SPPB to assess frailty. Although, this limits comparison, it seems that the current study is in line with those results. Hence, being frail according to the SPPB increased the risk for severe comorbidity almost fourfold to that seen in non-frailty hospitalized older adults in this study. Veronese et al. [26] showed that a low SPPB score predicted mortality. Thus, considering that the CCI is often used to predict mortality, results from the current study might be in agreement with that stated by Veronese et al. [26].

Lastly, an interesting finding from the current study is the synergetic effect observed between the nutritional status and performance-based physical tests. Indeed, according to handgrip strength and nutritional status, hospitalized older adults at risk of malnutrition or malnourished had higher CCI scores regardless of being fit or unfit according to handgrip strength. To our knowledge there is only one study carried out in a care home for veterans reporting a synergistic effect of malnutrition and low handgrip strength on 4-year all-cause mortality [23]. The authors reported that malnourished individuals with low handgrip strength were at 3.14 times higher risk of mortality [23], and that malnutrition was an independent risk factor for 4-year all-cause mortality [23]. In our study, in contrast, frail patients showed higher CCI scores despite being well-nourished or malnourished. To our knowledge there is no previous study combining nutritional status and frailty status, according to the SPPB threshold, of hospitalized older adults to compare with. Nevertheless, the results of the current study support frailty as a state of high vulnerability [45] and, thereby these inpatients aged ≥ 70 years might benefit from an exercise training program after hospitalization [46,47]. There is one recent study carried out in older adults admitted to hospital for acute coronary syndrome, where they

analyzed the incremental value from adding the MNA-SF as well as the SPPB to the model for predicting all-cause mortality [48]. The MNA-SF significantly improved the ability of the model to predict all-cause mortality, but the discrimination ability significantly improved with the addition of MNA-SF to the model with SPPB [48]. This needs to be further studied as the study was based on older adults with a specific characteristic (acute coronary syndrome), but it arises new insights to the use of the MNA-SF and the SPPB in clinical settings for predicting adverse clinical outcomes, and it reinforces our results.

To the best of our knowledge this is the first study carried out in hospitalized older adults (≥ 70 years old) examining the association between their nutritional status and physical function with the CCI score. Hence, nutritional status and physical function assessments were conducted by using the most widely easy-to-use tools recommended for geriatric hospitalized adults, the MNA-SF and handgrip strength and the SPPB, respectively. Another strength of the current study could be considered the large sample size (N = 597) as required for studies examining the prognosis of comorbidity [6]. However, some limitations should be recognised. First, the cross-sectional design of the study limits to determine any causality. Second, although the CCI is widely used in clinical settings, it was developed in a specific population different from the sample of the current study [32], and scores are often obtained from medical records, that although being more complete than other sources, might have added some bias recording some diseases [6]. Third, the reference percentiles for handgrip strength that were used might have limited the results [36]. Thus, future studies regarding handgrip strength and comorbidity risk will be required to contrast the results of the current study. Finally, this study cannot be extrapolated to other older adult population not meeting the inclusion criteria for this study and from other clinical settings or to community-dwelling older adults. So, future studies in population with different clinical characteristics should be conducted.

Conclusions

The current study confirms that malnutrition and poor physical function are associated with increased comorbidity (CCI) in hospitalized older adults aged 70 and older. Hence, being malnourished or frail increased the risk to be classified as at severe comorbidity (CCI). Both, along with other syndromes, are wide-spread conditions in older adults and often overlapped [49]. This hinders the identification of risk factors contributing to comorbidity and, finally mortality, within older adult population. However, the results in this study suggest that frailty, according to the SPPB frailty threshold, might be a major contributor to the CCI increase than the nutritional status in hospitalized older adults. Nevertheless, the current study reinforces the

use of the MNA-SF and the SPPB in geriatric hospital patients as they might help to predict poor clinical outcomes, and indirectly post-discharge mortality risk [50]. Thereby, including both tests into the routine clinical practice will help to better screen those patients at risk and will also permit to better monitor their evolution during and after hospitalization.

Conflict of Interest

Author Contributions

Funding

Acknowledgments

Abbreviations

CCI: Charlson Comorbidity Index

MNA-SF: Mini Nutritional Assessment-Short Form

SPPB: Short Physical Performance Battery

CVD: Cardiovascular Disease

COPD: Chronic Obstructive Pulmonary Disease

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FIGURE LEGENDS

Fig. 1 Flow diagram of participants

Fig. 2 Odd Ratios and 95% confidence intervals (95% CI) for being classified as at severe comorbidity risk (CCI ≥ 5, [32]) according to the nutritional status (MNA-SF) and different performance-based physical tests (handgrip and SPPB). Abbreviations: MNA-status: Mini Nutritional Assessment-status; SPPB-frailty threshold: Short Physical Performance Battery-frailty threshold. Unadjusted odds ratios. Nutritional assessment by the Mini Nutritional Assessment-Short Form questionnaire (scores ≤ 11 at risk of malnutrition or malnourished); handgrip unfit assessment according to Dodds et al. [36] percentiles (≤ P25 unfit); frailty assessment according to the Short Physical Performance Battery frailty threshold (scores ≤ 9 frail). Ref: scores > 11 normal nutritional status; > P25 fit; scores > 9 non-frail.

Fig. 3 Differences among comorbidity risk according to the nutritional status combined with several physical parameters. (A): nutritional status combined with fitness categories assessed by handgrip strength (kg) (≤ P25 unfit, [36]). (B): nutritional status combined with frailty categories (SPPB score ≤ 9 frail). Unadjusted analysis of variance (polynomial). a: $p < 0.005$; b: $p < 0.001$

Table 1. Characteristics of participants in the study by comorbidity risk according to the Charlson Comorbidity Index (CCI).

	N	Whole sample	N	Moderate risk of comorbidity	N	Severe risk of comorbidity	P
Age (years)	597	84.3 (6.8)	103	78.3 (5.8)	494	85.5 (6.3)	<0.001†
Female (N, %)	597	300, 50.3	103	59, 57.3	494	241, 48.8	0.117
Body mass (kg) [a]	583	67.2 (13.3)	100	69.5 (12.9)	483	66.7 (13.3)	0.050†
Number of drugs	597	7.2 (3.7)	103	5.1 (3.5)	494	7.7 (3.6)	<0.001†
Polypharmacy (N, %)	597	448, 75.0	103	51, 49.5	494	397, 80.4	< 0.001
Depression (N, %)	597	55, 9.2	103	14, 13.6	494	41, 8.3	0.145
Diseases							
Hypertension (N, %)	597	441, 73.9	103	64, 62.1	494	377, 76.3	< 0.05
CVD (N, %)	597	177, 29.6	103	4, 3.9	494	173, 35.0	< 0.001
COPD (N, %)	597	120, 20.1	103	5, 4.9	494	115, 23.3	< 0.001
Diabetes (N, %)	597	203, 34.0	103	12, 11.7	494	191, 38.7	< 0.001
Kidney disease (N, %)	597	107, 17.9	103	2, 1.9	494	105, 21.3	< 0.001
Hepatic disease (N, %)	597	13, 2.2	103	1, 1.0	494	12, 2.4	0.223
Neoplasia (N, %)	597	122, 20.4	103	0, 0.0	494	122, 24.7	< 0.001
Dementia (N, %)	597	23, 3.9	103	2, 1.9	494	21, 4.3	0.161
Parkinson (N, %)	597	19, 3.2	103	6, 5.8	494	13, 2.6	0.191
Nutritional Status							
MNA-SF score	597	10.0 (2.5)	103	10.9 (2.1)	494	9.8 (2.6)	<0.001†
Normal nutritional status (N, %)	597	205, 34.3	103	51, 49.5	494	154, 31.2	< 0.001
At risk of malnutrition (N, %)	597	288, 48.2	103	44, 42.7	494	244, 49.4	
Malnourished (N, %)	597	104, 17.4	103	8, 7.8	494	96, 19.4	
Physical Function							
Handgrip (kg) [b]	596	19.5 (8.2)	103	22.2 (8.8)	493	19.0 (8.0)	< .005†
SPPB total score [c]	591	5.4 (3.1)	102	7.2 (2.9)	489	5.0 (3.0)	< .001†
SPPB categorized							
0-3 (N, %)	591	188, 31.8	102	13, 12.7	489	175, 35.8	< 0.001
4-6 (N, %)	591	191, 32.3	102	29, 28.4	489	162, 33.1	
7-9 (N, %)	591	135, 22.8	102	30, 29.4	489	105, 21.5	
10-12 (N, %)	591	77, 13.0	102	30, 29.4	489	47, 9.6	

Note: Values are means and standard deviations unless otherwise is indicated. CVD: cardiovascular disease; COPD: chronic obstructive pulmonary disease; MNA-SF score: Mini Nutritional Assessment-Short Form score; SPPB total score: Short Physical Performance Battery total score.

*p refers to differences between patients at moderate and severe comorbidity risk analyzed by t test for independent samples in continuous variables and Chi-squared test for categorical variables. †means and standard deviations are presented for not transformed variables to ease interpretation, but *p* were obtained by t test for independent samples with logarithmically transformed continuous variables.

[a]Data were missing for 14 patients

[b]Data was missing for 1 patient

[c]Data were missing for 6 patients

Table 2. Linear regression of comorbidity risk assessed by the Charlson comorbidity index (CCI) with nutritional status and physical function by sex.

		Nutritional status			Physical function					
		MNA-SF (total score)			Handgrip (kg)			SPPB (total score)		
		β	Error and R^2	P	β	Error and R^2	P	β	Error and R^2	P
CCI (lineal)	Males	-0.125 IC 95% (-0.281 - -0.013)	0.068 / 0.016	<0.05	-0.185 IC 95% (-0.286 - -0.070)	0.055 / 0.034	<0.005	-0.200 IC 95% (-0.151 - -0.042)	0.028 / 0.040	<0.005
	Females	-0.154 IC 95% (-0.231- -0.036)	0.050 / 0.024	<0.05	-0.265 IC 95% (-0.266 - -0.110)	0.040 / 0.070	<0.001	-0.306 IC 95% (-0.175 - -0.082)	0.024 / 0.094	<0.001

Note: CCI: Charlson comorbidity index; MNA-SF (total score): Mini Nutritional Assessment-Short Form (total score); SPPB (total score): Short Physical Performance Battery (total score). Unadjusted linear regression tests. Linear regressions were calculated with the logarithmically transformed variables.

Figure 1
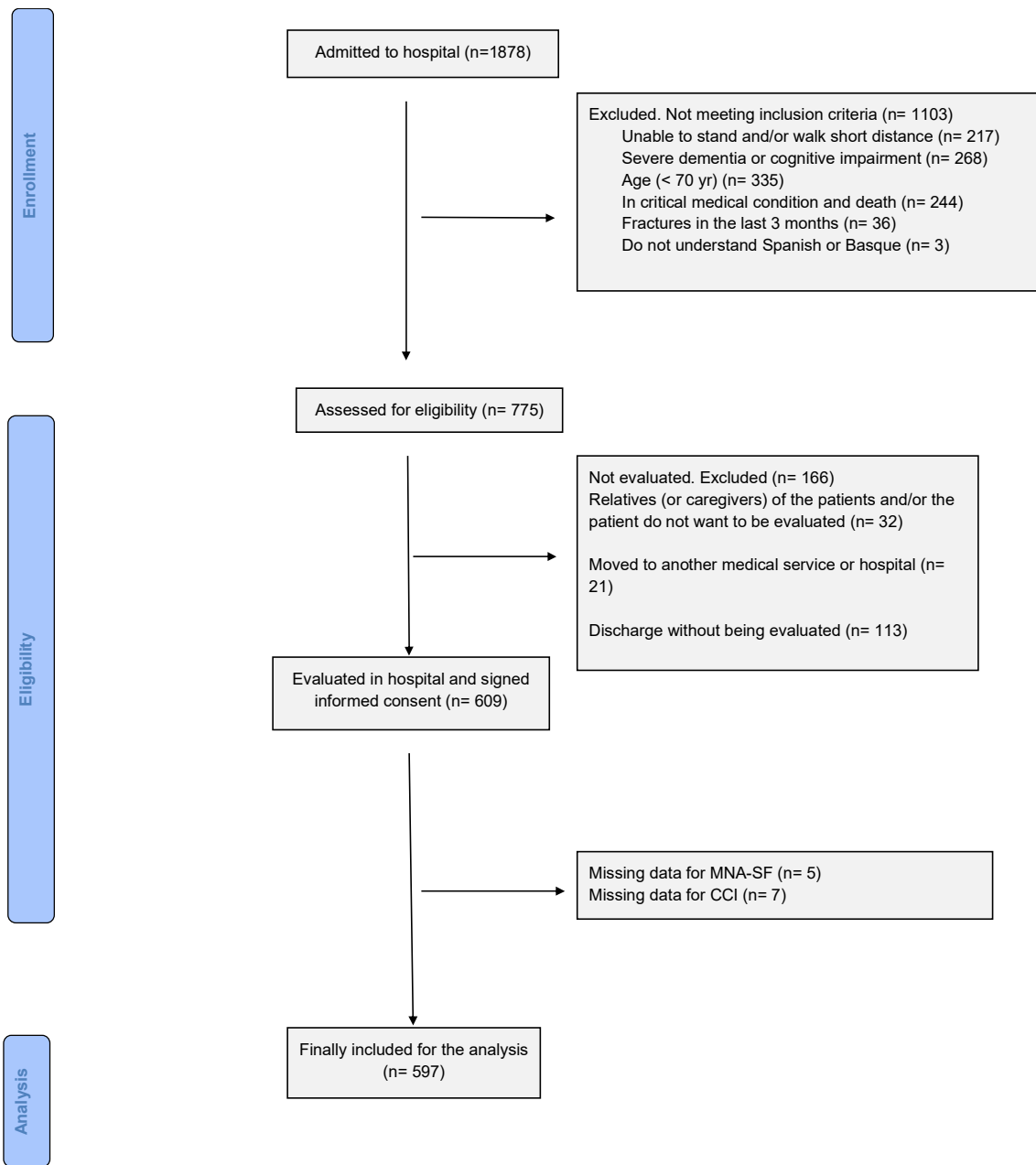
Figure 2

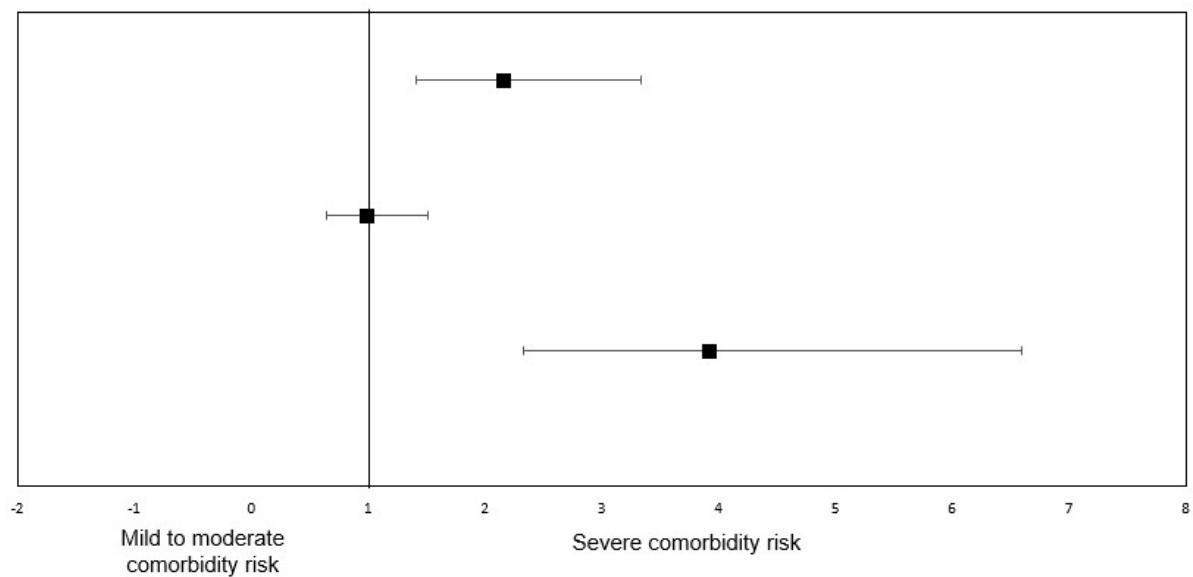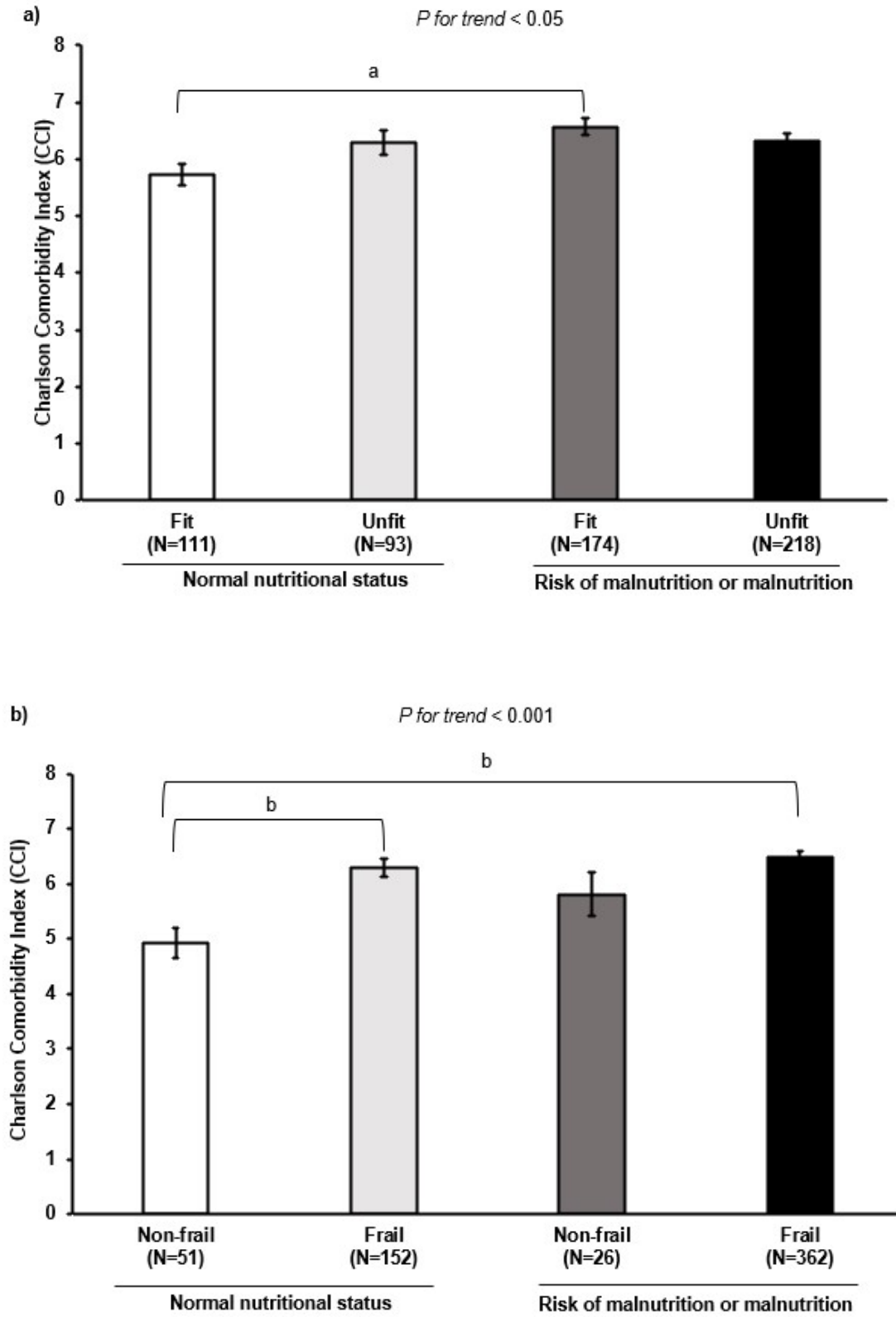| Nutritional and physical performance tests	OR (95% CI)	P
Risk of malnutrition or malnourished (MNA)	2.165 (1.408-3.331)	P < 0.001
Unfit (Handgrip)	0.988 (0.646-1.512)	P = 0.956
Frail (SPPB-frailty threshold)	3.918 (2.326-6.600)	P < 0.001

Figure 3

7.3 Study 3: Effect of Leucine-Enriched Whey Protein Supplementation on Physical Function in Post-hospitalized Older Adults Participating in 12-weeks of Resistance Training Program: A Randomized Controlled Trial

Effects of leucine-enriched whey protein supplementation on physical function in post-hospitalized older adults participating in 12-weeks of resistance training program: a randomized controlled trial.

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Abstract:

Age-related strength and muscle mass loss is further increased after acute periods of inactivity. To avoid this, resistance training has been proposed as an effective countermeasure, but the additional effect of a protein supplement is not so clear. The aim of this study was to examine the effect of a whey protein supplement enriched with leucine after resistance training on muscle mass and strength gains in a post-hospitalized elderly population. A total of 28 participants were included and allocated to either protein supplementation or placebo supplementation following resistance training for 12-weeks (2 days/week). Physical function (lower and upper body strength, aerobic capacity and the Short Physical Performance Battery (SPPB) test), mini nutritional assessment (MNA) and body composition (Dual X-ray Absorptiometry) were assessed at baseline and after 12-weeks of resistance training. Both groups showed improvements in physical function after the intervention ($p < 0.01$), but there were no further effects for the protein group ($p > 0.05$). Muscle mass did not improve after resistance training in either group ($p > 0.05$). In conclusion, 12-weeks of resistance training are enough to improve physical function in post-hospitalized elderly population with no further benefits for the protein supplemented group.

Keywords: elderly; aging; muscle mass; strength; resistance training; leucine; whey protein; protein supplementation

Introduction

Aging is characterized by a progressive decline in skeletal muscle mass and function defined as sarcopenia [1]. Sarcopenia is related to an increased risk of falling [1], fractures [2], physical disability [3] and mortality [4]. In healthy aging, muscle mass loss ranges from 3% to 8% per decade [5]. However, this decline is further emphasized by acute or chronic illness [6], inactivity [6] and inadequate protein and/or energy intake [7]. So, physical activity is proposed as an effective countermeasure to delay the age-related muscle mass loss [7]. Indeed, following a healthy lifestyle may help to prevent and reduce the consequences of age-related muscle mass loss [7].

A balanced protein metabolism is important to muscle mass accretion and maintenance [8]. Energy and protein intake are key nutritional factors to achieve protein balance [7]. However, due to several physiological and social factors, elderly people tend to reduce food intake and, in consequence, often fail to meet energy and protein requirements [9]. Likewise, protein-energy malnutrition is frequent in elderly patients [9]. Besides total daily protein intake [10], dietary protein quality and its anabolic potential have also received increased interest with the goal of optimizing skeletal muscle anabolism in the elderly [10,11].

Dietary protein quality depends on its digestibility, amino acid (AA) profile and AA availability [12,13]. Therefore, in studies aiming to optimize muscle mass among elderly people, whey protein (≈ 20g/day) is considered superior to other isolated protein sources [14,15]. Whey protein is also characterized for being a high leucine containing protein, which is the main precursor for activating muscle protein synthesis via mammalian target of rapamycin (mTOR) signalling [8,16]. A protein/AA source containing around 1.8-2.0g of leucine would be enough to activate post-exercise "leucine trigger", whereas in rested conditions a higher dose might be required in young adults [16]. Other authors [15], reported that 20g of whey protein enriched with 3g of leucine post-exercise resulted in a greater muscle protein synthesis rate in healthy older people.

Muscle mass accretion and strength gain depend on the synergistic effect of protein consumption and resistance training [8,16]. Protein ingestion close after exercise seems to increase exercise-induced muscle mass sensitivity to anabolism [8]. In a recent systematic review and meta-analysis [17] it was concluded that a combination of protein supplementation and resistance training led to positive effects on body composition, muscle volume and strength, and physical function in elderly people. In contrast, in people aged 70 years or older, it was shown that despite overall improvements from baseline for the majority of outcomes, there

were no significant differences between the group receiving protein/AA supplementation along with resistance training and the group with resistance training alone [18].

Acute periods of inactivity such as a hospital stay, accentuates age-related muscle mass loss [6]. After hospitalization older individuals are more vulnerable to develop any adverse event [19]. Then, early interventions to accelerate recovery and avoid hospital readmission will be important [20]. For example, implementing interventions that combine nutrition and physical exercise immediately after discharge [20].

In view of this growing interest, the objective of the present study was to examine the effect of a whey protein supplement enriched with leucine after resistance training on muscle mass and strength gains in a post-hospitalized elderly population. We hypothesized that elderly people after hospitalization may benefit most from the synergetic effect of protein supplementation and a resistance training session.

Methods

Study design

The Sarcopenia & Fragilidad-protein (S and F-PROT) study is a prospective, 24-weeks, single-blind, randomized, placebo-controlled clinical trial (ClinicalTrials.gov ID: NCT03815201). The study was conducted at the facilities of the Araba University Hospital in Vitoria-Gasteiz (North Spain), from September 2017 to July 2018. The Clinical Research Ethics Committee of the Araba University Hospital (CEIC-HUA: 2017-021) approved the study protocol (S&F-PROT) that complied with the revised ethical guidelines of the Declaration of Helsinki (revision of 2013). All participants were informed about the details of the research and signed an informed consent before their enrolment in the study.

The S and F-PROT project compared relative changes on functional capacity (muscular strength of upper and lower limbs, and aerobic capacity), body composition (lean mass and fat mass at whole body, arms, legs and trunk) and nutritional status between two groups following a resistance training intervention program with post-exercise supplementation (Protein-group) or without supplementation (Placebo-group).

Participants

Volunteers accessed the program after hospitalization at the internal medicine service of the Araba University Hospital, or by medical recommendation at the outpatient internal medicine speciality at the Araba University Hospital (**Figure 1**). Hospitalized patients older than

70 years old were first pre-screened for eligibility. All pre-screened participants met the following criteria: > 70 years old, a punctuation of ≥ 20 at the Mini Mental State Questionnaire (MMSE), fulfilled the criteria for sarcopenia diagnosis of the European Working Group on Sarcopenia in Older People, were able to walk alone or using a walking stick, a walking frame, or parallel walking bars, were able to understand the instructions or what had being said, and signed the informed consent. Patients were excluded for examination if they had any of the following exclusion criteria: history of chronic kidney disease, have suffered a heart attack in the last 3 months, been unable to walk, have suffered any fracture of the upper or lower limbs in the last 3 months, been suffering from severe dementia, a history of autoimmune neuromuscular disorders (for example, myasthenia gravis, Guillain-Barré syndrome, inflammatory myopathies) or amyotrophic lateral sclerosis, or refused to sign the informed consent.
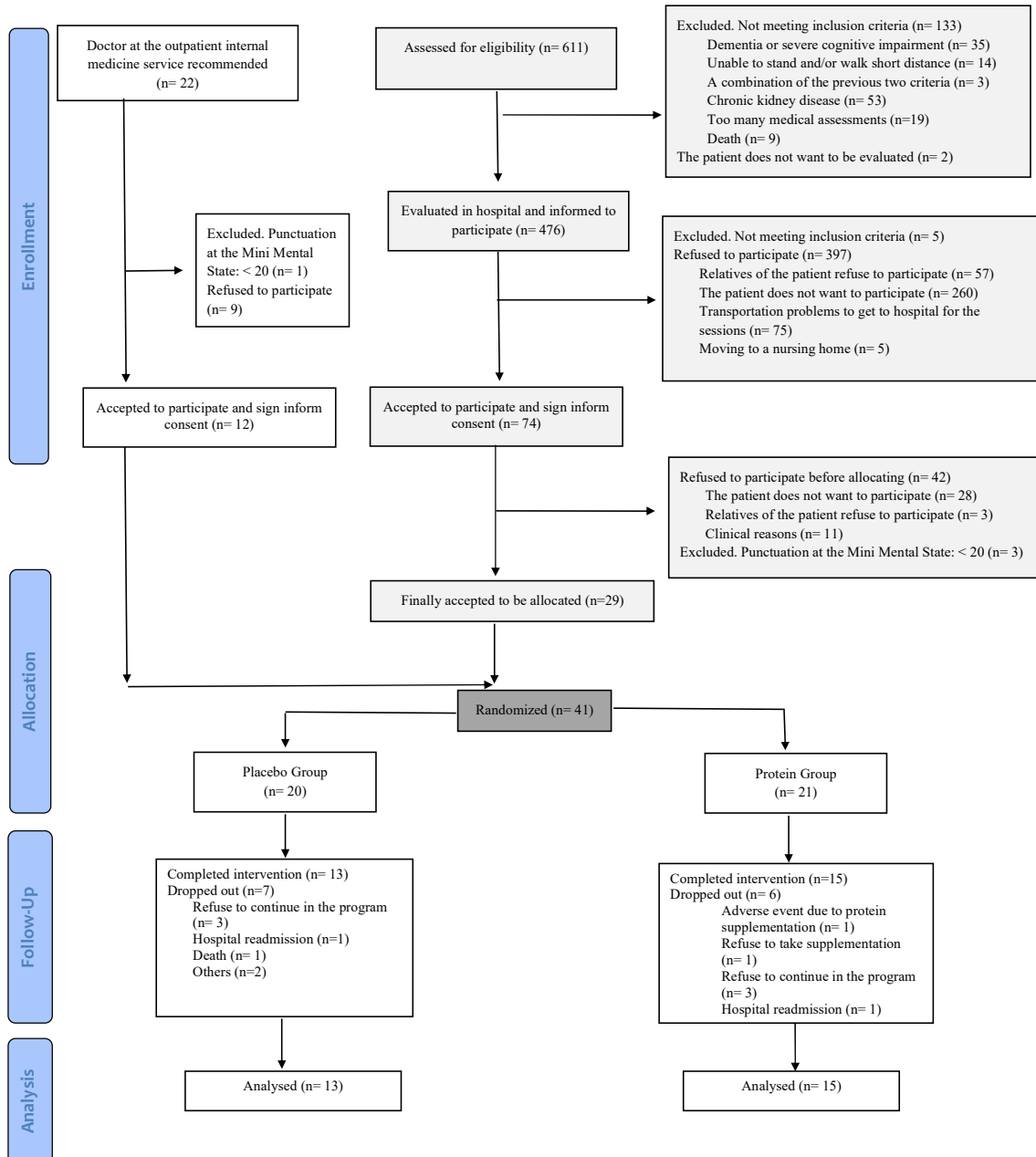
Figure 1. Flow Diagram of participants

Patients that were eligible for the intervention program were assessed for nutritional status (Mini Nutritional Assessment-Short Form (MNA-SF; Nestlé Nutrition Institute)) [21], physical function (Short Physical Performance Battery (SPPB) [22] and handgrip strength), frailty (a Spanish language version of the Fried test [23]) and cognitive function (Spanish validated version of the Pfeiffer test, the Short Portable Mental Status Questionnaire (SPMSQ) [24]) during their hospitalization. Patients were informed about the possibility of participating in an exercise training program after hospital discharge and an informed consent was given along with further

written information. After a recovery week, patients were cited for baseline physical function assessment before initiating the intervention program.

Many hospitalized patients did not meet inclusion criteria when assessing eligibility (21.8%) or refused to participate (66.6%) because of health issues, lack of interest in the physical exercise program, or had problems to get to hospital for the intervention sessions. As the hospital recruitment proved not to be enough for the intervention aims, the outpatient internal medicine service was chosen as an alternative recruitment source. Those patients at the outpatient internal medicine service potentially meeting inclusion criteria were informed by their doctor about the exercise intervention program. Thereafter, patients were cited for a first eligibility assessment with the investigation team. If participation criteria were met, patients were again cited a week after for baseline physical function assessment (Figure 1).

Randomization

Following baseline physical function assessment, participants were randomly allocated to one of the two intervention groups: Placebo-group or Protein-group. Participant stratification was based on gender to ensure equal allocation in both groups.

Supplementation and blinding

Placebo and protein supplements were delivered by the nutritionist in the first half hour following each training session. The protein supplement contained 20g of whey protein isolate (Davisco ®: BiPRO all-natural whey protein isolate, Eden Paririe, MN, USA) enriched with 3g of leucine (Nutricia, Madrid, Spain). The nutritional composition of both the placebo and protein supplement is shown in Table 1. The supplements were energy-matched and were flavoured with lemon flavour and solubilized in 150 mL of water. Only participants were blinded for supplementation. Supplements were stored in boxes and only the research team could identify them. All supplements were developed, prepared and stored in boxes by Laboratorium Sanitatis SL (Tecnalia Research and Innovation, Vitoria-Gasteiz, Spain).

Table 1. Nutritional composition of the protein and placebo supplements.

Nutritional composition	Protein supplement
B-lactoglobulin (g/bottle)	20
L-Leucine (g/bottle)	3
Sodium saccharin (g/bottle)	0.050
Sucralose (g/bottle)	0.030
Lemon flavour 654500 (g/bottle)	0.250
	Placebo supplement
Maltodextrin (g/bottle)	23
Hydroxyethylcellulose (g/bottle)	0.200
Lemon flavour 654500 (g/bottle)	0.250

Design of the resistance training program

Both groups followed a supervised resistance training program for 12 weeks. The program consisted of 1-hour sessions in two non-consecutive days per week. The first week of intervention was used for familiarization, and 1-RM (repetition maximum) estimation by the individual's functional capacity through Brzycki equation [25]. The load was then gradually increased during a month, and half exercises were performed at 50-65% of the estimated 1-RM. During the subsequent months load was increased until 70% of the estimated 1-RM was reached. Two sets were performed per exercise and load and maximum repetition for each exercise was personalized for each participant. All resistance training sessions were designed and supervised by a sport scientist with experience in resistance training for elderly.

All training sessions started with warm-up exercises (heel stand, calf raises, chair stand exercise and neck movements) and were followed by strengthening exercises of upper and lower limbs (arm-curl exercise with the participant in a seated position and personalized load, knee extension exercise with personalized load in a seated position, standing knee flexion with personalized load, side hip raise, standing hip extension and chair stand exercise). In the same resistance training session, some exercises for dynamic balance improvement were also practiced (side-by-side stand, semitandem stand, tandem stand, monopodal stand, timed up and go, stepping around obstacles and step up and down exercises). The session finished with 5 minutes of cool-down, consisting mainly of stretching exercises.

Outcome measures

Primary and secondary outcomes were assessed at baseline and after 12 weeks of intervention by the same trained researchers. Post-intervention measurements were scheduled within one week following the last exercise session.

Primary outcome: physical function

Physical function was assessed at baseline and at week 13 (once supervised intervention period was finished).

Physical function was assessed using a combination of tests. The tests used to assess lower and upper body strength and aerobic capacity were based on the Senior Fitness Test [26]. For lower and upper body strength, 30-Second Chair Stand Test and 30-Second Arm Curl Test were used, respectively. For upper body strength, isometric handgrip strength was also measured using a handled dynamometer (JAMAR® PLUS + Hand dynamometer). Aerobic capacity was assessed by the 6-minute walking test (6MWT). Hence, for physical function

assessment the SPPB test battery was also used [22]. This test includes the 4-meters walking speed test, the standing balance test (side-by-side stand, semi-tandem stand and tandem stand) and the time to rise from a chair five times test [22].

Secondary outcomes

Nutritional Assessment

A nutritionist completed all nutritional questionnaires along with the participant and/or participant's relative or caregiver. Participant's nutritional status was assessed using the MNA questionnaire (Nestlé Nutritional Institute) [27]. This questionnaire contains 18-items divided into 4 categories: anthropometric assessment, general assessment, short dietary assessment and subjective assessment [27]. Each answer has a numerical value contributing to the final punctuation. A maximum of 30 points can be obtained. Punctuation ranging from 24 to 30 reflects normal nutritional status, from 17 to 23.5 risk of malnutrition and a punctuation under 17 reflects malnutrition [27].

Body composition

Body fat, lean mass, bone mass, bone mineral density (BMD) and bone mineral content (BMC) were assessed by dual-energy X-ray absorptiometry (DXA; HOLOGIC, QDR 4500).

Body mass (OMRON HN-288, Digital Personal Scale, Barcelona, Spain) was measured barefoot following the standard protocols. Height was estimated using knee height determination (SECA 220, Hamburg, Germany) [28]. Body mass index (BMI) was calculated as body weight divided by height squared (kg/m^2). Waist circumference, hip circumference, calf circumference and mid-arm circumference were measured with a nonelastic tape (CESCORF, Porto Alegre, Brasil) following the protocol recommended by the International Society for the Advancement of Kinanthropometry (ISAK).

Biochemical parameters

Biochemical parameters were obtained from fasting venous blood samples in Ethylenediaminetetraacetic acid-containing tubes and in serum tubes. These tubes were immediately carried to the laboratory and EDTA-containing tubes were centrifuged at 1000 x g at 4ºC for 10 minutes whereas serum tubes were centrifuged 90 minutes after blood collection

at 1000 x g at 20ºC for 15 minutes. Serum albumin, prealbumin and creatinine were measured as protein malnutrition markers.

Statistical analysis

Baseline characteristics between groups (i.e., placebo *vs*. protein supplementation) were compared using independent Student t test.

Sample size estimation and power analysis was calculated for muscle mass increase. With a population size of 35 on each group, a significant alpha level of 0.05, and power > 80%, the range for a statistically detectable change in muscle mass will be 1.5-2kg with a standard deviation of 1.5-1.7kg.

Data analysis was performed following the per-protocol principle. Changes in primary and secondary outcomes were calculated as Post-intervention *minus* Pre-intervention values. Differences between the placebo and the protein groups (fixed factor) in changes on primary outcome and secondary outcomes were calculated by analyses of covariance adjusting with baseline values.

All statistical analyses were performed using the statistical software SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) with a level of significance of $\alpha = 0.05$. Data are expressed as means ± standard error of the mean.

Results

During the recruitment period, a total of 476 hospitalized patients were evaluated and invited to participate in the study. From them, only 74 (15.5%) accepted to participate, whereas the remaining 402 patients refused to participate (83.4%) or did not meet inclusion criteria (1.15%). Finally, from the 74 patients who accepted to participate, a total of 29 participants (39.2%) were randomized for the study. Overall, the 93.9% of the evaluated hospitalized patients did not participate in the study due to inclusion criteria or rejection to participate in the program. Regarding the recruitment from the outpatient internal medicine service, a total of 22 patients were recommended to participate. The 40.9% of these patients refused to enter the intervention program or did not meet inclusion criteria (4.5%), whereas the 54.54% accepted. In total, 41 patients were randomized for the intervention program, 20 entered the Placebo-group and 21 the Protein-group. From the allocated participants, 13 did not complete the 12 weeks of the intervention program (7 from the Placebo-group and 6 from the Protein-group). The main reason for being dropped out from the study in both groups was that participants refused to continue in the program (15.0% of the randomized patients in the Placebo-group and 14.3% in the Protein-group). In the Protein-group an adverse event was reported with protein supplementation regarding itchy throat and difficulties to inhale, whereas another participant refused to take the protein supplement, so both participants were dropped out from the study (Figure 1). Baseline characteristics of the recruited participants can be found in Supplemental Table S1.

Table 2 shows baseline characteristics of participants. There were no statistically significant differences in body composition and nutritional status variables between groups at baseline. However, within the physical function parameters, the protein-group walked significantly more meters in the 6MWT at baseline ($p < 0.05$). In contrast, the Protein-group showed significantly greater lean mass on the legs (%) than the Placebo-group ($p < 0.05$).

Table 2. Characteristics of participants completing the study (intend-to-treat analyses).

	N	Placebo group	N	Protein group	P
Age (years)	13	81.7 (6.45)	15	82.9 (5.59)	0.607
Women (N, %)	13	7 (53.8)	15	7 (46.7)	0.717
Body mass (kg)	13	75.9 (17.95)	15	68.0 (11.43)	0.188
BMI (Kg/m^2)	13	30.8 (6.53)	15	27.4 (3.50)	0.110
Physical Function					
Handgrip (kg/body mass)	13	0.3 (0.09)	15	0.4 (0.09)	0.063
SFT chair stand test 30sec	13	10.6 (4.17)	15	12.3 (2.97)	0.229
SFT arm curl test 30sec	13	13.5 (5.22)	15	16.3 (3.92)	0.137
SFT 6MWT (m)	13	314.8 (139.36)	15	411.5 (80.40)	0.040
SPPB total punctuation	13	8.7 (2.36)	15	10.1 (1.58)	0.089
SPPB 5Squat	13	14.7 (6.85)	15	12.2 (2.86)	0.232
Body composition					
Waist to hip ratio	13	1.00 (0.07)	15	0.98 (0.09)	0.459
Lean mass arms (kg)	13	2.3 (0.67)	15	2.3 (0.44)	0.897
Lean mass legs (kg)	13	6.8 (1.70)	15	6.4 (1.08)	0.441
Lean mass trunk (kg)	13	23.0 (4.83)	15	21.5 (3.89)	0.380
Total lean mass (kg)	13	45.2 (9.85)	15	42.3 (6.63)	0.391
Fat mass arms (%)	13	2.6 (0.96)	15	2.4 (0.77)	0.545
Fat mass legs (%)	13	5.8 (1.85)	15	5.4 (1.84)	0.603
Fat mass trunk (%)	13	17.1 (3.87)	15	14.9 (3.03)	0.124
Total fat mass (%)	13	35.4 (8.05)	15	32.1 (6.84)	0.259
Nutritional Status					
MNA score	13	23.1 (3.82)	15	24.5 (2.11)	0.273
Normal nutritional status (N, %)	13	4 (30.8)	15	11 (73.3)	0.064
At risk of malnutrition (N, %)	13	8 (61.5)	15	4 (26.7)	
Malnourished (N, %)	13	1 (7.7)	15	0 (0)	
Biomarkers					
Creatinine (mg/dl)	10	1.1 (0.48)	15	0.9 (0.35)	0.401
Albumin (g/dl)	13	4.0 (0.39)	15	4.0 (0.31)	0.994
Prealbumin (mg/dl)	12	22.2 (6.63)	14	23.3 (4.31)	0.613

BMI: body mass index; MNA score: Mini Nutritional Assessment score; SFT chair stand test 30sec: Senior Fitness Test chair stand test 30sec; SFT arm curl test 30sec: Senior Fitness Test arm curl test 30sec; SFT 6MWT (m): Senior Fitness Test 6-minute Walking Test (m); SPPB total punctuation: Short Physical Performance Battery total punctuation; SPPB 5Squat: Short Physical Performance Battery 5Squat. Values are means and standard deviations.

Effects of the intervention on primary outcomes: physical function

Both groups showed improvements over time in all the physical function tests ($p < 0.01$), except for the handgrip strength test (**Table 3**). However, we did not observe any significant difference between groups in any of the measured physical function tests (Table 3)

Table 3. Body composition, nutritional status and physical function in elderly patients before (Pre) and after (Post) their participation in the resistance exercise intervention program plus protein supplementation (Protein group) or placebo (placebo group) (analyses *per protocol*).

	| Placebo group			| Protein group			| Differences between groups		
	N	Pre	Post	*P*	N	Pre	Post	*P*	Δ Placebo	Δ Protein	*P*
Primary outcome											
Physical function											
Handgrip (kg/body mass)	13	0.3 (0.09)	0.3 (0.09)	0.775	15	0.4 (0.09)	0.4 (0.09)	0.651	0.0 (0.03)	-0.0 (0.06)	0.971
SFT chair stand test 30sec	13	10.6 (4.17)	13.5 (4.59)	0.003	15	12.3 (2.97)	14.4 (3.22)	< 0.001	2.8 (2.79)	2.1 (1.53)	0.480
SFT arm curl test 30sec	13	13.5 (5.22)	21.9 (4.66)	< 0.001	15	16.3 (3.92)	23.5 (4.53)	< 0.001	8.4 (5.74)	7.2 (4.86)	0.724
SFT 6min WT (m)	13	314.8 (139.36)	375.0 (128.39)	0.002	15	411.5 (80.4)	455.1 (81.77)	0.005	60.2 (53.67)	43.6 (51.2)	0.959
SPPB total score	13	8.7 (2.36)	10.3 (1.89)	0.001	15	10.1 (1.58)	11.3 (0.96)	0.002	1.6 (1.39)	1.2 (1.21)	0.634
SPPB 5Squat	13	14.7 (6.85)	10.6 (3.67)	0.005	15	12.2 (2.86)	10.0 (2.81)	0.004	-4.1 (4.32)	-2.2 (2.4)	0.491
Secondary outcomes											
Body composition											
Body mass (kg)	13	75.9 (17.95)	75.6 (18.31)	0.621	15	68.0 (11.43)	68.3 11.07)	0.500	-0.3 (2.24)	0.3 (1.60)	0.471
BMI (kg/m^2)	13	30.8 (6.54)	30.7 (6.64)	0.575	15	27.4 (3.5)	27.5 (3.37)	0.453	-0.3 (2.24)	0.3 (1.60)	0.493
Waist to hip ratio	13	1.00 (0.07)	1.00 (0.08)	0.818	15	0.98 (0.09)	0.96 (0.08)	0.255	-0.0 (0.06)	-0.0(0.05)	0.400
Lean mass arms (kg)	13	2.3 (0.67)	2.3 (0.41)	0.937	15	2.3 (0.44)	2.2 (0.41)	0.049	0.0 (0.36)	-0.1 (0.24)	0.088
Lean mass legs (kg)	13	6.8 (1.7)	6.9 (1.45)	0.630	15	6.4 (1.08)	6.5 (1.04)	0.260	0.1 (0.64)	0.1 (0.34)	0.756
Lean mass trunk (kg)	13	23.0 (4.83)	22.6 (4.47)	0.212	15	21.5 (3.88)	21.7 (3.61)	0.198	-0.4 (1.21)	0.2 (0.67)	0.128
Total lean mass (kg)	13	45.2 (9.85)	44.7 (8.54)	0.545	15	42.3 (6.63)	42.5 (6.61)	0.458	-0.4 (2.52)	0.2 (1.02)	0.611
Fat mass arms (%)	13	2.6 (0.96)	2.6 (0.85)	0.808	15	2.4 (0.77)	2.3 (0.92)	0.291	-0.0 (0.56)	-0.1 (0.41)	0.575
Fat mass legs (%)	13	5.8 (1.85)	5.9 (2.07)	0.165	15	5.4 (1.84)	5.5 (1.69)	0.506	0.2 (0.45)	0.1 (0.46)	0.549
Fat mass trunk (%)	13	17.1 (3.86)	16.7 (3.31)	0.448	15	14.9 (3.03)	15.7 (2.61)	0.061	-0.4 (1.86)	0.7 (1.31)	0.297
Total fat mass (%)	13	35.4 (8.05)	35.2 (7.53)	0.728	15	32.1 (6.84)	32.7 (6.64)	0.092	-0.2 (1.91)	0.6 (1.31)	0.357
Nutritional status											
MNA score	13	23.1 (3.8)	25.3 (2.2)	0.010	15	24.5 (2.1)	26.2 (1.6)	0.019	2.2 (2.6)	1.7 (2.5)	0.512
Normal nutritional status (N. %)	13	4(30.8)	9(69.3)	0.123	15	11(73.3)	14(93.4)	0.533			
At risk of malnutrition (N. %)	13	8(61.6)	4(30.8)		15	4(26.7)	1(6.7)				
Malnourished (N. %)	13	1(7.7)	0		15	0	0				
Biomarkers											
Creatinine (mg/dl)	10	1.1 (0.48)	1.1 (0.37)	0.664	15	0.9 (0.35)	0.9 (0.32)	0.595	0.0 (0.21)	0.0 (0.14)	0.438
Albumin (g/dl)	13	3.9 (0.39)	4.1 (0.31)	0.189	15	3.9 (0.31)	4.0 (0.26)	0.499	0.1 (0.22)	0.0 (0.15)	0.331
Prealbumin (mg/dl)	12	22.2 (6.63)	20.5 (4.48)	0.221	14	23.3 (4.31)	21.3 (4.17)	0.019	-1.6 (4.36)	-1.9 (2.77)	0.916

SFT chair stand test 30sec: Senior Fitness Test chair stand test 30sec; SFT arm curl test 30sec: Senior Fitness Test arm curl test 30sec; SFT 6MWT (m): Senior Fitness Test 6-minute Walking Test (m); SPPB total punctuation: Short Physical Performance Battery total punctuation; SPPB 5Squat: Short Physical Performance Battery 5Squat; BMI: body mass index; MNA score: Mini Nutritional

Assessment score. Values are means and standard deviations. *P indicates statistical differences between Pre and Post values (paired t-Student test). Δ placebo indicates the difference between Pre and Post values in the Placebo group; Δ Protein indicates the difference between Pre and Post values in the Protein group. P indicates statistical significance between Δ placebo and Δ Protein (ANOVA).

Effects of the intervention on secondary outcomes

We did not observe any significant difference on body composition measurements within groups at the end of the intervention, except for lean mass on arms within the protein group ($p < 0.05$, Table 3). There were no significant differences in any of the body composition variables between the two groups (Table 3).

The MNA scoring improved significantly within both groups after the intervention program ($p < 0.05$, Table 3). However, we did not observe any significant difference on changes in MNA score between groups ($p < 0.5$, Table 3).

Among serum markers of protein malnutrition, creatinine and albumin concentrations did not significantly change over time in either group (Table 3). Prealbumin concentrations significantly decreased in the Protein-group ($p < 0.05$, Table 3). Nevertheless, there were no significant differences on changes in protein nutritional status serum biomarkers between groups (Tables 3).

Discussion

The current study aimed to examine the additional effect of a leucine-enriched protein supplementation on physical function, skeletal muscle mass and nutritional status after resistance training in a post-hospitalized elderly population. Results do not show further beneficial effects with protein and leucine-enriched supplementation after 12 weeks of resistance training (2 sessions/week) for any of the measured variables. These findings suggest that protein supplementation might not be determinant to see improvements in muscle mass and strength, and/or the time period of the intervention was not enough to see significant results.

It is well-established that resistance training is an effective countermeasure to combat age-related skeletal muscle mass and strength loss [6,29]. It is proposed as a primary intervention for sarcopenia [30], frailty [31], malnutrition [32] and other geriatric syndromes [7]. Our results are in line with these guidelines, according to physical function measurements as both groups show improvement after resistance training.

Resistance training stimulates muscle protein synthesis [33]. To take advantage of this anabolic stimuli, we considered protein supplementation as a complementary strategy following resistance training. In line with studies supporting this strategy [15,34], the protein-group received 20g of whey protein enriched with 3g of leucine after each session twice per week. However, there were no further benefits on physical function for the protein-group in this study.

This is in contrast with some [34-36], but not all [37,38] previous studies. A recent systematic review [18], concluded that protein/AA supplementation did not further improve muscle strength in older subjects following a resistance training program. Nevertheless, both groups showed significant improvements in physical function parameters, except for handgrip strength. This result was also seen in the study carried by Leenders et al. [29], where they suggested that handgrip strength is not a clinically relevant and/or valid measure to evaluate changes in muscle function in response to a resistance training program in the elderly.

We observed no changes in body composition after the intervention in either group. Again, we did not see further benefits with protein supplementation. One previous study with participants aged 82 years reported a limited muscle plasticity that further limited strength gains in response to a progressive resistance training program [39]. So, our results in a population with the same average age (82 years) underscore the limited capacity to hypertrophy as we age [40]. Furthermore, when looking for studies regarding muscle mass and strength gains along with protein supplementation, among many of them the target adult population are younger than age 80 [34,36,41-43]. The same issue can be seen in recent systematic reviews and meta-analysis, where most of the included studies are based in younger population [44,45]. However, this blunted anabolic response might be overcome, or at least minimized, if adequate interventions are designed [46]. It seems that the protein synthesis capacity of the muscle is preserved up to very old age in response to anabolic stimuli [33].

There is still much controversy regarding protein supplementation in the elderly population due to poor compliance, high heterogeneity and underpowered studies evident from meta-analysis and systematic reviews [17,18, 45]. Studies underlying protein supplementation as an effective measure to increase resistance training induced adaptations were based on short-term metabolic studies [15,34,47]. Conversely, dietary intervention studies, where long-term protein supplementation have been examined, have failed to observe measurable gains in skeletal muscle mass in the elderly population [35,37,38]. Tieland et al. supplemented the protein-group with 15g of protein at breakfast and lunch for 24 weeks and they reported that protein intake in this group increased to more than 25g with each meal (daily protein intake increased from 1.0 ± 0.1 to 1.4 ± 0.1g/kg body mass/day) [35]. However, they did not observe measurable gains in skeletal muscle mass for the protein-group as baseline protein intake was already high [35].

Contrary to Tieland et al. [35], participants in our study entered the interventional program after hospitalization where they had suffered an acute phase of illness and inactivity,

and it would be reasonable to think that the protein-group should have benefit more from the resistance training along with protein supplementation. However, during the post-hospitalization period exists an acquired, transient period of vulnerability known as post-hospital syndrome, where the nutritional requirements are higher than normal to reverse this acute situation [19,48]. It has been suggested to increase dietary protein to 1.2 - 1.5g/kg body mass/day during acute illness or up to 2.0g/kg body mass/day in severe situations [48]. Thus, it could be speculated that if the protein supplementation protocol used in the study of Tieland et al. was not enough to see gains in muscle mass [35], the one applied in our study neither. It is probably that participants in our study were below the dietary protein recommendation set for acute phases or that the dietary treatment on this study did not increase the daily protein intake to have an effect. However, in contrast to Tieland et al. [35], the protein-group in our study was supplemented immediately after resistance training in order to overcome any daily protein deficiency and take advantage of the increased exercise-induced anabolic stimuli. In a recent study conducted in mobility-limited older adults, the protein-group was supplemented after resistance training with 20g of whey protein three times per week for six months [42]. Englund et al. concluded that protein supplementation improved body composition [42]. However, the target population in the study conducted by Englund et al. had a total SPPB score ≤ 9 [42], whereas the protein group in our study had a baseline total SPPB score ≥ 9. This suggests that our participants were in better physical condition and that had better body composition, although they entered the program after an acute illness phase. So, there might be different hypotheses to assess why further benefits were not seen with protein supplementation in muscle mass accretion. The acute illness phase might had been the limiting factor which had increased the nutritional requirements of our participants, not only for protein needs but also daily energy intake. So, it could be that until all nutritional requirements are met, just twice per week protein supplementation after resistance training is not enough for muscle mass accretion. Conversely, as baseline protein intake was not reported, it could be that it was already within the protein dietary recommendation and that our participants in the protein-group were, after all, within an acceptable range of physical and health condition or that the deviation for protein intake from baseline was not sufficient to induce muscle mass gains [49].

The latter hypothesis might be more probable because muscle mass did not increase with protein supplementation, but even more important did not decrease after resistance training in either group. This suggests that participants in this study were within the RDA for protein, otherwise negative protein balance would have occurred hampering muscle mass maintenance [50], and it also reinforces the idea that healthy elderly people with an adequate

daily protein intake might not benefit from increased protein content [51]. Furthermore, at baseline participants in the protein-group had an average punctuation of 24.5 in the MNA, which is considered a normal nutritional status. In turn, within the placebo-group the average punctuation was 23.1, so they were almost at risk of malnutrition according to the MNA. But, following the resistance training program the mean punctuation in the placebo-group increased to 25.3, achieving a good nutritional status. These results suggest that nutritional requirements were within an acceptable range among both groups leading to improve physical function variables and maintenance of muscle mass in both groups. Nevertheless, it is worth to mention that protein supplementation was not prescribed to participant body-weight, which could have benefit more muscle mass accretion. It is also important to highlight that the rate of protein turnover in older adults is slower, so improvements in strength and physical performance are often seen before measurable changes in skeletal muscle mass become apparent [48]. Thus, it is unlikely to observe significant changes in muscle mass after only 12 weeks of twice per week supervised intervention. For muscle mass accretion, a positive protein balance must be achieved over time along with resistance exercise [50]. Indeed, it might be that the overall volume employed in our resistance training program was not enough to see gains in muscle mass [52]. In addition, the sample size in this study might not be enough to see beneficial effects in muscle mass accretion following post-training protein supplementation [18, 49, 52].

Limitations and strength

The current study has several limitations. Daily protein and energy intake of participants were not controlled, we can merely speculate that probably total daily protein intake, the protein distribution among meals, the protein supplementation and/or the duration of our interventional program were not enough to increase muscle mass in this study. In addition, we assessed changes in body composition by DXA and with this methodology differences smaller than 1.0kg are not detectable [35]. The recruitment has been lower than what we expected with only 20 and 21 participants in the Placebo-group and in the Protein-group, respectively. However, the dropout rate has been high with only 13 and 15 participants completing the intervention study in the Placebo-group and in the Protein-group, respectively. When designing the study, it was proposed that 35 participants should be included to each group to see detectable improvements in muscle mass. So, this might be the main reason for not having seen significant improvements in muscle mass after the intervention. Another limitation is that the study was single-blinded and not double blinded. One of the strengths of the study is that to the

best of our knowledge this is the first randomized study including post-hospitalized elderly adults in a resistance training program along with protein supplementation.

Conclusions

This study reinforces resistance training as a fundamental early intervention strategy to maintain muscle mass and increase gains in physical function parameters in post-hospitalized elderly adults. Thus, 12-weeks of supervised resistance training with one-hour session among 2 days/week seems enough to enhance strength and physical function variables in post-hospitalized elderly adults. However, it does not clarify the additional benefits of a protein supplementation.

The elderly population is a very heterogenic group, so future directions should focus on conducting studies among the different subgroups with special needs. There is a need to assess which might be the optimum length of an interventional study including resistance exercise and supplementation to induce gain in muscle mass and strength. Specially, there is a growing interest in stablishing the characteristics of the best protein supplementation protocol and difference between healthy older adults and older adults with an acute or chronic disease and/or with one or more conditions of the geriatric syndrome. It would also be interesting for future studies to add muscle biopsies as direct measurements for muscle mass hypertrophy.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Characteristics of the recruited participants in the study (intend-to-treat analysed).

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Supplemental Table S1. Characteristics of the recruited participants in the study (intend-to-treat analyses).

	N	Total	N	Placebo group	N	Protein group	*P*
Age (years)	41	82.1(5.89)	20	81.2 (6.14)	21	82.9 (5.67)	0.354
Women (N, %)	41	22 (53.7)	20	10 (50)	21	12 (57.1)	0.647
Body mass (kg)	40	72.4 (15.6)	19	77.5 (17.02)	21	67.8 (12.92)	0.052
BMI (Kg/m^2)	40	29.1 (5.22)	19	31.1 (5.83)	21	27.4 (3.95)	0.025
Physical Function							
Handgrip (kg/body mass)	40	0.3 (0.09)	19	0.3 (0.09)	21	0.4 (0.09)	0.021
SFT chair stand test 30sec	41	10.6 (4.39)	20	9.9 (4.91)	21	11.2 (3.86)	0.358
SFT arm curl test 30sec	41	14.9 (4.98)	20	14.5 (5.09)	21	15.4 (4.95)	0.557
SFT 6MWT (m)	41	352.1 (119.45)	20	315.3 (131.26)	21	387.2 (97.61)	0.056
SPPB total punctuation	41	9.1 (2.4)	20	8.7 (2.4)	21	9.5 (2.36)	0.278
SPPB 5Squat	38	13.2 (4.99)	18	13.7 (6.17)	20	12.8 (3.76)	0.602
Body composition							
Waist to hip ratio	40	0.99 (0.09)	19	1.01 (0.07)	21	0.97 (0.1)	0.120
Lean mass arms (kg)	41	2.4 (0.63)	20	2.5 (0.75)	21	2.3 (0.49)	0.310
Lean mass legs (kg)	41	6.7 (1.43)	20	6.9 (1.58)	21	6.5 (1.27)	0.382
Lean mass trunk (kg)	41	22.2 (4.39)	20	23.2 (4.73)	21	21.2 (3.91)	0.142
Total lean mass (kg)	41	43.9 (8.62)	20	45.8 (9.68)	21	42.2 (7.27)	0.189
Fat mass arms (%)	40	2.5 (0.91)	19	2.7 (0.93)	21	2.3 (0.88)	0.170
Fat mass legs (%)	40	5.8 (1.74)	19	5.9 (1.72)	21	5.6 (1.78)	0.499
Fat mass trunk (%)	40	15.9 (3.53)	19	17.3 (3.28)	21	14.5 (3.29)	0.011
Total fat mass (%)	40	33.8 (7.39)	19	36.1 (7.25)	21	31.8 (7.08)	0.066
Nutritional Status							
MNA score	39	23.5 (3.0)	19	23.0 (3.5)	20	24.0 (2.4)	0.311
Normal nutritional status (N, %)	39	20 (48.7)	19	7 (35)	20	13 (61.9)	0.160
At risk of malnutrition (N, %)	39	18 (43.9)	19	11 (55)	20	7 (33.3)	
Malnourished (N, %)	39	1 (2.4)	19	1 (5)	20	0 (0)	
Biomarkers							
Creatinine (mg/dl)	37	1.0 (0.39)	17	1.1 (0.46)	20	0.9 (0.33)	0.228
Albumin (g/dl)	40	4.0 (0.32)	20	4.0 (0.36)	20	4.0 (0.28)	0.806
Prealbumin (mg/dl)	37	23.0 (5.52)	18	23.6 (6.48)	19	22.5 (4.54)	0.535

BMI: body mass index; MNA score: Mini Nutritional Assessment score; SFT chair stand test 30sec: Senior Fitness Test chair stand test 30sec; SFT arm curl test 30sec: Senior Fitness Test arm curl test 30sec; SFT 6MWT (m): Senior Fitness Test 6-minute Walking Test (m); SPPB total punctuation: Short Physical Performance Battery total punctuation; SPPB 5Squat: Short Physical Performance Battery 5Squat.

7.4 Study 4: Effects of Resistance Training Intervention Along with Leucine-Enriched Whey Protein Supplementation on Sarcopenia and Frailty in Post-Hospitalized Older Adults: Preliminary Findings of a Randomized Controlled Trial

Resistance training intervention along with leucine enriched whey protein supplementation on sarcopenia and frailty in post-hospitalized older adults: preliminary findings of a randomized controlled trial

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Abstract

Resistance training along with protein supplementation are expected to exert the greatest stimulus to counteract muscle wasting conditions. Myokines might play a key role, but this needs to be elucidated yet. The aim of this study (NCT03815201) was to examine the effects of a resistance training program with post-exercise leucine-enriched-protein supplementation on sarcopenia and frailty status, and on plasma myokine concentrations of post-hospitalized older adults. A total of 41 participants were included to this 12-weeks of resistance training intervention and randomized either to the Placebo-group or the Protein-group. Sarcopenia, frailty, body composition and blood-based myokines were measured at baseline and after 12 weeks. Both groups improved their physical performance ($p < 0.005$) and frailty ($p < 0.07$) following the resistance training intervention, but without any difference between groups. Myokine concentrations did not change after the intervention in either group. Changes in myostatin concentrations were associated with greater improvements in Appendicular Skeletal Muscle Mass at the end of the intervention ($p < 0.05$). In conclusion, the implementation of resistance training programs after hospitalization in older adults should be prioritized to combat sarcopenia and frailty immediately. Results regarding myostatin should be taken as preliminary findings.

Keywords: elderly; strength training; muscle wasting; muscle mass; myokine; myostatin; protein; leucine; whey protein; hospital

Introduction

Muscle mass loss is a widely known consequence of aging [1]. This progressive loss of muscle mass along with impaired muscle strength and function is known as sarcopenia [2]. Sarcopenia might derive in physical frailty [2]. However, the cumulative decline that occurs throughout life in multiple physiological systems might also result in frailty [3]. Likewise, frailty is considered a geriatric syndrome and it might be present independently of sarcopenia [3]. Both conditions, sarcopenia and frailty, are characterized by a decline in muscle strength and poor physical function [3,4]. This can be further accelerated by physical inactivity [5].

Physical activity is proposed as one of the most effective countermeasures to address muscle wasting related conditions [5–7]. Specifically, resistance exercise training provides the necessary stimulus to promote muscle hypertrophy through several signaling pathways [8,9], and it is proposed that this might be further emphasized by protein supplementation in older adults [10]. Hence, leucine is considered the main precursor for activating muscle protein synthesis [11]. Likewise, it has been suggested that leucine enriched protein supplementation might help to improve sarcopenia in older adults [12]. Muscle contraction triggers the release of myokines that influence those signalling pathways, and thereby muscle growth [13].

Recent studies have examined how myokines act in response to exercise training in older adults to see whether the obtained benefits could be mediated by myokines' response or not. Hofman et al. concluded that the improvements observed after resistance training program could have been mediated by follistatin induced blocking of the muscle degradation pathways rather than by lower circulating levels of myostatin [14]. Nevertheless, there is still much controversy regarding the response of both myokines to exercise, but specially according to myostatin, some reporting a reduction [15,16] while other showing a higher myostatin concentrations after an exercise intervention program [17,18]. Regarding irisin response to exercise training, it seems that aerobic and resistance training [19-21] stimulate irisin release increasing its circulating levels in older adults, but there are also studies showing no changes [22]. The controversy shown in those studies is in accordance with those studies examining the role of these myokines in muscle wasting-related conditions [23-25].

There is growing interest to know if those myokines might be contributing to the muscle weakness seen with aging [26], and to see if they could serve as blood-based biomarkers [27,28]. However, there are still many aspects that need to be studied before, such as myokines' dynamic in response to exercise and aging. Therefore, the aim of this study was to examine the effects of a resistance training program along with post-exercise enriched protein supplementation on

sarcopenia and frailty status as well as on plasma myokine concentrations of post-hospitalized older adults. We hypothesized that those older adults with sarcopenia and/or frailty might benefit most from resistance training along with leucine enriched protein supplementation after hospitalization, and that those improvements might be highlighted by changes on plasma myokine concentrations. Thus, our second hypothesis was that changes in myokine plasma concentrations might predict muscle mass improvements.

Materials and Methods

Study design

The Sarcopenia & Fragilidad-PROT (S&F-PROT) study is a 24-weeks, single-blind, randomized, placebo-controlled clinical trial (ClinicalTrials.gov ID: NCT03815201) conducted from September 2017 to July 2018 at the facilities of the Araba University Hospital in Vitoria-Gasteiz (North Spain). The study protocol (S&F-PROT) was approved by the Clinical Research Ethics Committee of the Araba University Hospital (CEIC-HUA: 2017-021) and by the Ethics Committee for Research with Biological Agents of the University of the Basque Country (CEIAB: M30/2018/201), that complied with the revised ethical guidelines of the Declaration of Helsinki (revision of 2013). More detailed information as well as the main effects of the project have been published elsewhere [29] and can be found in Table S1.

Overall, the study consisted of a supervised resistance training intervention program without (Placebo-group) or with (Protein-group) post-exercise supplementation. Briefly, the protein supplement contained 20 g of whey protein isolate enriched with 3 g of leucine. Participants were randomly assigned in a parallel design (1:1 ratio, stratified by gender) to one of the two intervention groups. Randomization was done by the researchers involved in the intervention, so blinding was not possible for them, just participants were blinded for allocation (intervention group). Informed consent was obtained from all subjects involved in the study. Thereby, written informed consent has been obtained from the patients to publish this paper. All of them were informed about the details of the research. The program consisted of 12-weeks of supervised training sessions followed by a period of 12-weeks non-supervised. However, the current study is based on the first 12 weeks of supervised training. Participants attended the training program two non-consecutive days per week (1-hour sessions). Detailed information regarding supplementation and the design of the resistance training program can be found elsewhere [29]. Briefly, the resistance training program was tailored to each participant based on 1-RM (repetition maximum) estimation, and load was then gradually increased until 70% of the estimated 1-RM was reached at the end of the intervention [29]. Sessions started with

warm-up exercises followed by strengthening exercises of upper and lower limbs and finished with 5 min of cool-down [29]. Each exercise was performed twice, and load and maximum repetition vary according to each participant [29].

Participants

Participants were recruited during their hospitalization at the internal medicine service or by medical recommendation at the outpatient internal medicine specialty at the Araba University Hospital [29]. The inclusion and exclusion criteria as well as the recruitment process have been described before along with the participants flow-diagram [29]. As described before [29], the hospital recruitment process was not enough for the intervention aims, so the outpatient internal medicine service was chosen as an additional recruitment source. From hospital a total of 29 older inpatients finally accepted to participate in the intervention program, whereas from the outpatient internal medicine service a total of 12 older adults accepted. As a result, a total of 41 older adults were randomized to either of the intervention groups. However, only 28 participants completed the intervention program (13 in the Placebo-group and 15 in the Protein-group) (Figure S1). Briefly, participants were ≥ 70 years old post-hospitalized patients or geriatric outpatients with no medical contraindication nor physical and cognitive impairment for their participation in the intervention program.

Measurements

All measurements were performed at baseline and after 12-weeks of intervention (at week 13) by the same trained researchers.

Sarcopenia assessment

Sarcopenia was assessed following the proposed algorithm for case-finding by the European Working Group on Sarcopenia in Older People 2 (EWGSOP2) in the revised European consensus on definition and diagnosis for sarcopenia [2]. For the current study, participants were first screened for muscle strength according to the handgrip strength cut-off points, and those with low muscle strength were then assessed for muscle quantity based on Appendicular Skeletal Muscle Mass (kg) cut-off points to confirm sarcopenia [2]. Handgrip strength (kg) was measured using a handled dynamometer (JAMAR® PLUS + Hand dynamometer) in a seating position as proposed by Roberts et al. [30]. The test was performed twice alternating each hand, the highest value was chosen and used for analysis.

Frailty assessment

It has been suggested that the Short Physical Performance Battery (SPPB) might help to identify frailty in elderly population [31,32]. The SPPB assessment methodology has been published elsewhere [33], and it consists of 3 subtests (balance, gait speed and chair stand) that contribute to the SPPB total score, which ranges from 0 to 12 [33]. The SPPB frailty threshold has been proposed at ≤ 9 points [31,32]. Thus, participants in the current study with a score ranging from 0 to 9 in the SPPB were classified as "frail" whereas those scoring ≥ 10 were classified as "non-frail".

Body composition assessment

Body mass (kg) (OMRON HN-288, Digital Personal Scale, Kyoto, Japan) was measured barefoot following the standard protocols and height was estimated using knee height determination (SECA 220, Hamburg, Germany) [34]. For body mass index (BMI) estimation, body weight was divided by height squared (kg/m^2). Calf circumference (cm) was measured by nonelastic tape (CESCORF, Rio Grande do Sul, Brazil) on the left side following the instructions of the Mini Nutritional Assessment questionnaire [35]. All measures were performed twice and the averaged was used for analysis.

Body fat, lean mass, fat free mass and bone mass were assessed by dual-energy X-ray absorptiometry (DXA; HOLOGIC, QDR 4500, Bedford, MA, USA). Likewise, the sum of lean mass from both arms and legs was used to assess Appendicular Skeletal Muscle Mass (kg) and this was divided by height squared to assess Appendicular Skeletal Muscle Mass Index (kg/m^2). Similarly, Fat Mass Index was calculated as total fat mass divided by height squared (kg/m^2) and Fat Free Mass Index as total fat free mass divided by height squared (kg/m^2).

Blood-based biomarkers

Biochemical parameters were obtained from fasting venous blood samples in Ethylenediaminetetraacetic acid (EDTA)-containing tubes and in serum tubes a week after the last training session. All tubes were immediately carried to the laboratory. The EDTA-containing tubes were centrifuged at 1000g at 4ºC for 10 minutes whereas serum tubes were centrifuged 90 minutes after blood collection at 1000g at 20ºC for 15 minutes. The obtained serum aliquots from the participants were stored at -80ºC for further analysis. Myokines concentrations were quantified by commercial enzyme-linked immunosorbent assays (ELISA), following manufacturers' protocol. Serum myostatin (ng/ml) and follistatin (ng/ml) were measured using GDF-8/Myostatin and Follistatin Quantikine ELISA Kits, respectively (R&D Systems Inc.,

Minneapolis, MN, USA). Serum irisin (μg/ml) concentration was measured using a Irisin ELISA kit (AdipoGen Life Sciences, San Diego, CA, USA). For measurement of biomarkers the quantification was done by spectrophotometry with FLUOstar OPTIMA Microplate reader (ThermoFisher Scientific, Waltham, MA, USA) and Optima Control software version 2.20 (BMG, LABTECH, Ortenberg, Germany).

Statistical analysis

The current study is a secondary analysis. Indeed, sample size estimation and power analysis was calculated for muscle mass increase (i.e, the primary outcome of the primary study), [29]. It was estimated that with a population size of 35 on each group, a significant alpha level of 0.05, and power >80%, the range for a statistically detectable change in muscle mass will be 1.5-2kg with a standard deviation of 1.5-1.7kg.

Data analysis was performed following the per-protocol principle. Raw scores from each variable were winsorized (when needed) to limit the influence of the outliers (i.e., extreme values). In the current study the variables referring to myostatin baseline concentration and to changes in myostatin concentration were winsorized. The winsorization consists in replacing high/low values (percentile <1st or percentile >99th values) for the closest (highest/lowest) valid value (1st or 99th percentile) [36]. To test our main hypothesis changes in sarcopenia, frailty, body composition and blood-based biomarkers measurements were calculated as Post-intervention *minus* Pre-intervention values (Δ= post-pre). Paired sample *t*-test was used for continuous variables, whereas McNemar test was used for categorical variables. Analyses of covariance was done to examine differences in changes in continuous variables (dependent variables) using intervention group as fixed factor (i.e., the Placebo-group and the Protein-group) and adjusted for baseline values.

As there were not significant differences according to muscle mass parameters nor sarcopenia and frailty statuses between both groups at the end of the intervention program, we could not test our secondary hypothesis. However, we explored the potential role of myokines on muscle mass independently of the intervention group. For this aim, linear regressions were performed between changes in myokines concentrations (independent variable) and changes in muscle mass parameters (dependent variable) at the end of the intervention, each muscle mass parameter was adjusted for its baseline value. Among those myokines concentrations that showed significant association with any parameter of muscle mass, additional statistical analysis was conducted. Likewise, an analysis of covariance was done to compare the difference in myostatin concentration between those participants that had gained *vs*. those that had not

gained muscle mass at the end of the intervention adjusted by myostatin baseline concentration.

All statistical analyses were performed using the statistical software SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) with a level of significance of α = 0.05.**Results**

Table 1 shows baseline characteristics of participants by intervention group.

Table 1. Characteristics of participants in the study at baseline.

	N	Total	N	Placebo group	N	Protein group
Age (years)	41	82.1 (5.89)	20	81.2 (6.14)	21	82.9 (5.67)
Women (N, %)	41	22, 53.7	20	10, 50	21	12, 57.1
Body mass (kg)	40	72.4 (15.6)	19	77.5 (17.02)	21	67.8 (12.92)
Height (m)	40	1.6 (0.1)	19	1.6 (0.1)	21	1.6 (0.1)
BMI (Kg/m^2)	40	29.1 (5.22)	19	31.1 (5.83)	21	27.4 (3.95)
Fat mass index (kg/m^2)	40	10.1 (3.63)	19	11.4 (3.81)	21	8.9 (3.12)
Fat Free mass index (kg/m^2)	40	18.4 (2.46)	19	19.0 (3.09)	21	17.8 (1.56)
Calf circumference (cm)	40	35.6 (4.41)	19	36.3 (4.94)	21	34.9 (3.88)
Sarcopenic assessment						
Handgrip strength (kg)	41	25.3 (7.63)	20	24.5 (7.16)	21	26.1 (8.14)
Appendicular Skeletal Muscle Mass (kg)	41	18.1 (3.98)	20	18.7 (4.55)	21	17.5 (3.35)
Appendicular Skeletal Muscle Mass Index (kg/m^2)	40	7.2 (1.15)	19	7.5 (1.41)	21	7.1 (0.83)
Sarcopenic (N, %)	41	6, 14.6	20	3, 15.0	21	3, 14.3
Frailty assessment						
SPPB total score	41	9.1 (2.40)	20	8.7 (2.43)	21	9.5 (2.36)
Frail (N, %)	41	21, 51.2	20	13, 65.0	21	8, 38.1
Blood based biomarkers						
Myostatin (ng/ml)	40	3.3 (2.03)	20	3.5 (2.39)	20	3.1 (1.64)
Follistatin (ng/ml)	40	2.9 (1.26)	20	3.0 (1.46)	20	2.8 (1.06)
Follistatin to myostatin ratio	40	1.2 (0.95)	20	1.3 (1.08)	20	1.2 (0.82)
Irisin (µg/ml)	30	8.2 (3.96)	14	9.3 (4.17)	16	7.4 (3.66)

Data are presented as mean (standard deviation) unless other is indicated. BMI: body mass index. SPPB: Short Physical Performance Battery.

Effects of the intervention on sarcopenia and frailty status

Although both groups improved their physical performance according to the SPPB total score (all $p < 0.005$, **Table 2**), only the Placebo-group showed statistically significant improvement in the prevalence of frailty according to the SPPB threshold. Indeed, the number of frail participants declined from 9 to 3 after the intervention only in the Placebo-group ($p < 0.05$, Table 2). In the Protein-group, although it was not significant, 5 participants improved their frailty status following the intervention ($p = 0.063$, Table 2). In contrast, non-statistically significant improvements were seen for either of the intervention groups according to sarcopenia status (all $p > 0.05$, Table 2). Hence, there were not statistically significant differences

between groups regarding sarcopenia and frailty changes after the intervention program (all *p* > 0.05, Table 2).

Effects of the intervention on body composition and blood-based biomarkers

Table 2 shows that there were not statistically significant differences in body composition variables and myokines concentrations within each of the intervention groups nor between the two groups at the end of the intervention (all *p* > 0.05, Table 2).

Table 2. Body composition, nutritional status and physical function in elderly patients before (Pre) and after (Post) their participation in the resistance exercise intervention program plus protein supplementation (Protein group) or placebo (placebo group) (analyses *per protocol*).

		Placebo group				Protein group				Differences between groups		
	N	Pre	Post	p*	N	Pre	Post	p*		Δ Placebo	Δ Protein	p†
Sarcopenic assessment												
Handgrip strength (kg)[§]	13	24.8 (7.63)	24.5 (7.32)	0.704	15	26.9 (6.85)	26.6 (6.50)	0.699		-0.2 (2.28)	-0.4 (3.66)	0.883
Appendicular Skeletal Muscle Mass (kg)	13	18.3 (4.65)	18.5 (3.60)	0.681	15	17.3 (2.81)	17.3 (2.78)	0.787		0.2 (1.64)	-0.0 (0.77)	0.282
Appendicular Skeletal Muscle Mass Index (kg/m^2)	13	7.4 (1.50)	7.5 (1.16)	0.561	15	6.9 (0.64)	6.9 (0.66)	0.794		0.1 (0.63)	-0.0 (0.30)	0.150
Sarcopenic (N, %)	13	2, 15.4	2, 15.4	1.000	15	3, 20.0	2, 13.3	1.000		0.0, 0.0	-1, 6.7	1.000
Frailty assessment												
SPPB score total[§]	13	8.7 (2.36)	10.3 (1.89)	**0.001**	15	10.1 (1.58)	11.3 (0.96)	**0.002**		1.6 (1.39)	1.2 (1.21)	0.634
Frail (N, %)	13	9, 69.2	3, 23.1	**0.031**	15	6, 40.0	1, 6.7	0.063		-6, 46.2	-5, 33.3	0.700
Body composition												
Fat mass index (kg/m^2)	13	11.1 (4.35)	11.1 (4.67)	0.843	15	9.0 (3.02)	9.1 (2.81)	0.418		-0.0 (0.64)	0.1 (0.55)	0.460
Fat Free mass index (kg/m^2)	13	19.0 (3.29)	18.9 (2.80)	0.524	15	17.8 (1.37)	17.9 (1.45)	0.375		-0.2 (0.94)	0.1 (0.40)	0.731
Calf circumference (cm)	13	36.1 (5.28)	36.2 (5.29)	0.545	15	35.2 (3.64)	35.5 (3.44)	0.138		0.1 (0.82)	0.3 (0.81)	0.621
Blood based biomarkers												
Myostatin (ng/ml)	13	3.5 (2.8)	3.1 (1.85)	0.444	15	3.0 (1.85)	2.9 (1.45)	0.938		-0.3 (1.55)	- 0.0 (1.29)	0.799
Follistatin (ng/ml)	13	3.1 (1.26)	3.3 (1.73)	0.482	15	2.8 (1.08)	2.9 (1.49)	0.447		0.3 (1.36)	0.2 (0.87)	0.816
Follistatin to myostatin ratio	13	1.4 (1.09)	1.9 (2.29)	0.381	15	1.3 (0.91)	1.5 (1.58)	0.370		0.4 (1.79)	0.2 (0.90)	0.720
Irisin (µg/ml)	13	9.3 (4.3)	7.9 (2.99)	0.161	15	7.5 (3.76)	7.7 (3.57)	0.814		-1.4 (3.31)	0.2 (3.85)	0.624

Data are presented as mean (standard deviation). §Data from Amasene et al. (2019) [29]. *P indicates statistical differences between Pre and Post values by paired t-Student test (continuous variables) and McNemar test (categorical variables). Δ placebo indicates the difference between Pre and Post values in the Placebo group (Δ= post-pre); Δ Protein indicates the difference between Pre and Post values in the Protein group (Δ= post-pre). †P indicates statistical significance between Δ placebo and Δ Protein analyzed by analysis of covariance (continuous variables) or chi square test (categorical variables) adjusted for baselines values.

Association of changes in myokines' concentration with changes in muscle mass parameters following the intervention program

Table 3 shows the associations between changes in serum concentration of each measured myokine and the respective changes in muscle mass variables. Higher increases in myostatin concentrations at the end of the intervention were significantly associated with greater improvements in Appendicular Skeletal Muscle Mass (kg) (β = 0.319, *p* = 0.048, Table 3). Exploratory analyses showed that those participants that gained muscle mass following the intervention program were those that had greater, but non-significant changes in myostatin concentrations (Figure S2).

Table 3. Associations of changes in myokines with changes in muscle mass parameters after the intervention.

	Δ Handgrip strength (kg)		Δ ASMM (kg)		Δ ASMMI (kg/m²)		Δ FFMI (kg/m²)	
	β	*p*	β	*p*	β	*p*	β	*p*
Δ Myostatin (ng/ml)	0.043	0.819	0.319	0.048	0.256	0.128	0.243	0.165
Δ Follistatin (ng/ml)	-0.014	0.941	0.097	0.561	0.061	0.716	0.238	0.157
Δ Follistatin to Myostatin ratio	-0.055	0.771	0.066	0.693	0.049	0.771	0.128	0.455
Δ Irisin (μg/ml)	0.101	0.590	-0.084	0.615	-0.075	0.659	-0.130	0.445

β: standardized beta coefficient. ASMM: Appendicular Skeletal Muscle Mass; ASMMI: Appendicular Skeletal Muscle Mass Index; FFMI: Fat Free Mass Index. Linear regression tests adjusted for baseline values of each muscle mass parameter.

Discussion

This study aimed to examine if a 12-week resistance training program along with leucine-enriched protein supplementation after each training session (2 sessions/week) could add further benefits for post-hospitalized older adults (≥ 70 years old) for improving their frailty and sarcopenia status as well as exercise-induced myokines blood concentrations. The main finding of the current study is that the addition of leucine enriched whey protein to the resistance training program did not add any significant improvement to frailty and sarcopenia status. Moreover, no differences between groups were observed in blood-based biomarkers analyses.

The beneficial effects of resistance training on sarcopenia were not reflected on the sarcopenia status of the participants in this study. Following the EWGSOP2 algorithm, sarcopenia was confirmed based on handgrip strength (kg) and Appendicular Skeletal Muscle Mass (kg) cut-offs [2]. We previously suggested that 12-weeks of resistance training might have not been enough to see significant improvements in muscle mass measurements [29]. In this line, a meta-analysis conducted by Borde et al. concluded that 50-53 weeks were needed to observe effects on muscle mass in healthy older adults [37]. Hence, if besides this we considered

the acute effects of hospitalization on this population [5,38,39], the time scheduled in the current study was far from been optimal to observe significant improvements in muscle mass measurements. The sarcopenia criteria chosen for the current study might have influence on the non-significant findings, as the number of patients diagnosed with sarcopenia is lower with EWGSOP2 compared with other screening criteria [40,41]. Overall, it might be that due to these factors along with the multifactorial nature of sarcopenia [42] and the small sample size, the current study failed to observe any beneficial effects of resistance training on sarcopenia status. Hence, the number of those diagnosed with this condition was small. In contrast, according to frailty status, more than half of the older adults in the Placebo-group and the 40% in the Protein-group were frail at baseline. Performance within the SPPB was significantly improved in both intervention groups regardless of protein enriched supplementation. This improvement in performance did not represent a significant improvement in the frailty status of older adults in the Protein-group, but it did in the Placebo-group. Nevertheless, it is worth mentioning that, although non-significant, 5 of those frail older adults at baseline in the Protein-group were not frail after the intervention program. Again, the small sample size might have hampered to observe a significant effect of resistance training on frailty status in this intervention-group. Their performance in the SPPB improved significantly, so it might be suggested that with a greater sample size their frailty status would have been improved accordingly or that, as suggested for sarcopenia, more time ($\geq$ 12 weeks) is needed to observe the beneficial effects in frailty status. Nevertheless, these results highlight the efficacy of resistance training programs on improving physical performance of older adults, and thereby preventing the onset or progression of frailty on its early stages [43].

In the current study, the concentrations of myostatin, follistatin and irisin did not significantly change after the intervention program in neither of the groups nor between both groups (Placebo-group *vs*. Protein-group). The results published by Hofmann et al. are not in line with the results we observed for follistatin, but they are with those observed for myostatin levels showing no significant changes after the intervention [14]. However, other authors have reported significant decreases [15,16] or increases [17,18] on serum myostatin levels. According to irisin, our results are in line with Hecksteden et al., showing no effects of the intervention program on its serum levels [22].

Follistatin and irisin, both known for their anabolic effects [13], did not show any association with either of the measured muscle mass parameters in the current study. Surprisingly, myostatin showed a significant association with Appendicular Skeletal Muscle Mass (kg) suggesting that the change in myostatin concentrations (Post –Pre values of myostatin

concentrations) might predict or reflect the change in Appendicular Skeletal Muscle Mass (kg) (Δ ASMM (kg)) following the intervention program. Indeed, we also observed that those older adults that increased, although non significantly, Appendicular Skeletal Muscle Mass (kg) and Fat Free Mass Index (kg/m^2) after the intervention program were those that had the greatest change in myostatin concentration. Arrieta et al. did not observe a significant association between the increase in lean mass and myostatin concentrations following a multicomponent physical exercise intervention [18]. Despite this, they observed that improvements in physical parameters after the intervention were positively associated with higher myostatin levels in men [18]. In addition, in the Vienna Active Ageing Study it was observed that lower levels of myostatin at baseline were associated with a smaller increase in muscle mass or even muscle mass loss [14]. These results might have two possible explanations. On the one hand, as proposed by other authors [18,44], it could be that in light of the anabolic stimulus exert by the resistance training program, myostatin serum levels might have increased to restrain unlimited muscle mass growth acting as a chalone. On the other hand, as it has been suggested before, myostatin might be required for myogenesis to take place, despite being a negative regulator of it [45]. Thus, in line with our results, it could be that myostatin might have some implication in muscle mass gains, albeit following a resistance training program in older adults. By contrast, one could also speculate that simply, the effects at cellular level might not correspond with a direct decrease in serum levels of myostatin, albeit in the short-term [46]. For example, it could be that myostatin gene expression is suppressed and/or that its actions, such as decreasing mTORC1 signalling pathways [47], are blocked due to the stimuli of resistance training favoring muscle mass growth. However, the expected decrease in myostatin levels might take longer time to occur. Thus, just measuring serum myostatin levels might lead to mis-leading conclusions. Nevertheless, these are just speculations as the sample size in the current study was small and does not permit to reach conclusive statements.

Strength and limitations

Although our results, regarding myostatin's role, suggest a research line to follow, we should not omit the small sample size as a limitation of the current study. There are still many aspects that need to be clarify, as the sex-interaction. Some authors have suggested that myostatin might have a homeostatic role in males while in females might contribute to the age-related muscle mass loss [25]. But how these opposed roles of myostatin affects to the response to an anabolic stimulus within each gender needs to be stablished yet. The time-point chosen in this study for the collection of blood samples (1 week after finishing the training program) should be considered as a limitation too. Although, it is not well-stablished the time-point at which

serum myokine concentration might represent exercise-induced myokine released by muscle, it would have been more accurate if we had measured them between 24-72 hours after the last training session. Nevertheless, a recent meta-analysis showed that myostatin gene expression was down regulated in long-term within the skeletal muscle [48]. So, it might be speculated that the increase observed in myostatin serum concentration in this study was due to the acute response of muscle mass to resistance training. However, it is just a speculation and this as well as the time course of other myokines should be studied in future studies. Thus, future studies, regarding these issues and others, with larger sample sizes are needed before general conclusions are made. The small sample size in this study was also stated as an important limitation explaining the lack of significant improvements in muscle mass after the intervention [29] along with the inability of DXA to detect differences smaller than 1.0 kg [49]. These limitations might have also limited us to observe significant improvements in the sarcopenia status of participants. The single-blinded characteristic of the study should also be taken as a limitation. One of the strengths of the current study is that, to our knowledge, this is the first randomized controlled trial including older adults immediately after hospitalization in a resistance training program with post-exercise leucine-enriched protein supplementation.

Conclusions

This study reinforces resistance training as a primary countermeasure to combat and/or prevent frailty in post-hospitalized older adults. Although, significant improvements in frailty status were only observed in one intervention group, older adults in both groups significantly improved their physical performance regardless of the protein supplementation. We consider this an important point to highlight, as we observed that with 12-weeks of resistance training we start to observe beneficial effects on physical performance of older post-hospitalized adults. Likewise, we suggest that, if prolonged, sarcopenia and frailty status will improve accordingly. Future studies should be conducted to stablish the minimum length needed to observe significant improvements in sarcopenia and frailty status of post-hospitalized older adults. Studies examining the effective dose, frequency and meal distribution of protein supplementation are also needed in these muscle wasting conditions. Hence, due to their multifactorial nature it might be that protein supplementation should be accompanied by other nutritional supplements [50].

In addition, our results regarding myostatin's role should be taken as preliminary findings that need to be proven in future studies with larger sample sizes. Future studies with larger sample sizes need to be conducted to understand how myostatin responds to training

stimuli at cellular as well as at systemic level and if those responses correspond with the training outcomes observed in different contexts.

we would also like to thank the Araba University Hospital in Vitoria-Gasteiz for providing their facilities.

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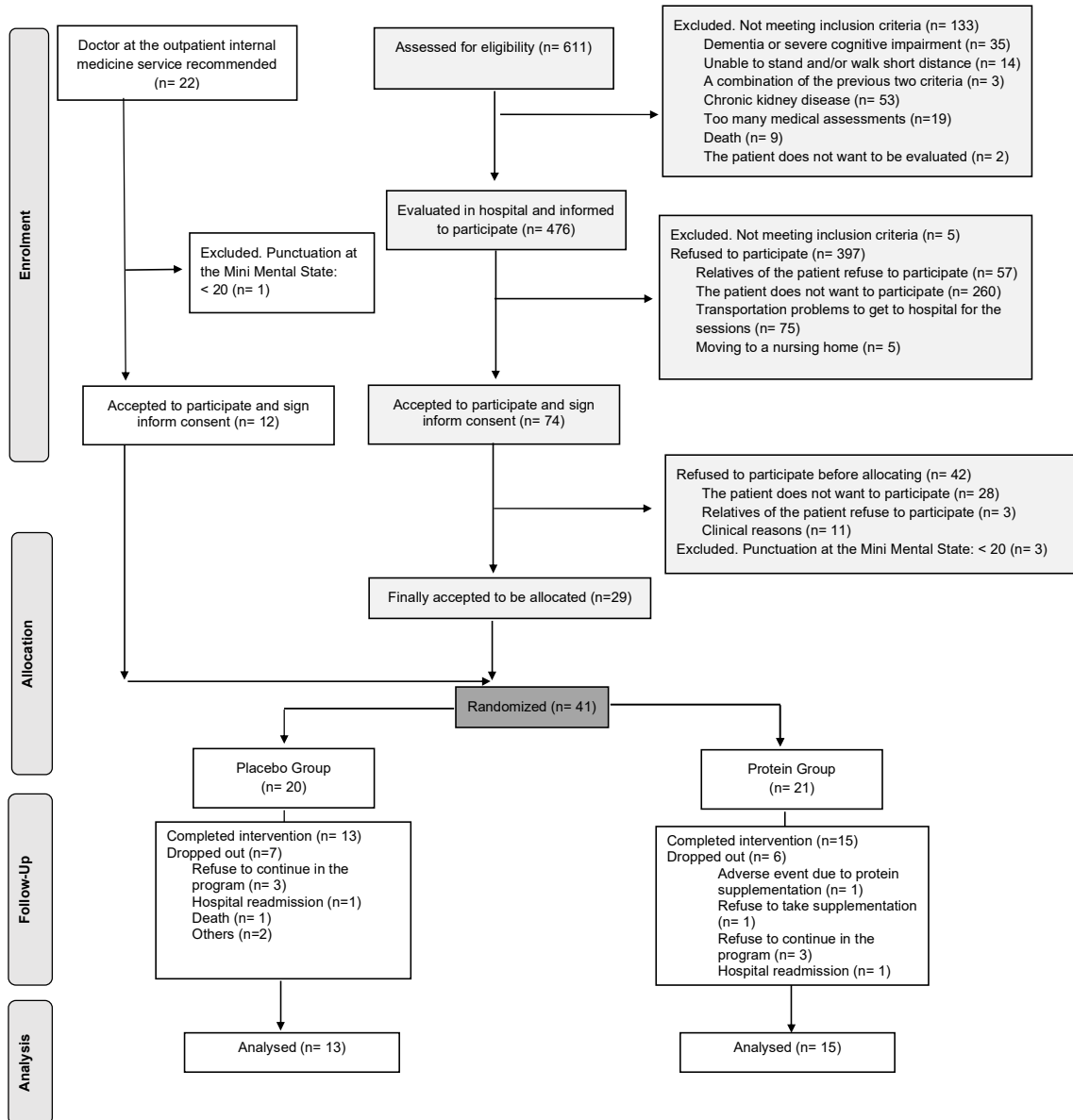
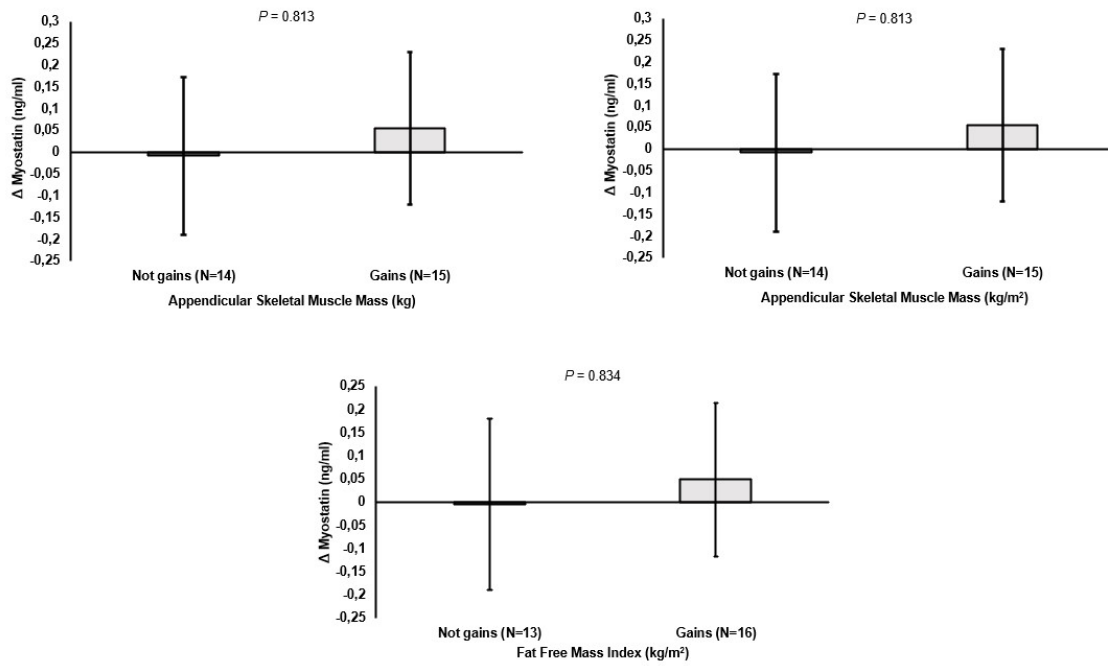**Figure S1.** Flow diagram of participants

Figure S2. **Comparison of the difference in myostatin concentration between participants with muscle mass gains vs. not muscle mass gains after the intervention program.** Analysis of covariance adjusted for myostatin baseline concentration.

SECTION	ITEM #	CONSORT-SPI 2010	CONSORT-SPI 2018	REPORTED ON PAGE#
TITLE AND ABSTRACT				
	1a	Identification as a randomised trial inthe title		Page 1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for Abstracts)	Refer to CONSORT extension for social and psychological intervention trial abstracts	Page 1, abstract
INTRODUCTION				
Background and Objectives	2a	Scientific background and explanationof rationale		Page 1-2, line 46-82
	2b	Specific objectives or hypotheses	If pre-specified, how the intervention was hypothesized to work	Page 2, line 79-87
METHODS				
Trial Design	3a	Describe of trial design (such as parallel, factorial), including allocationratio	If the unit of random assignment is not the individual, please refer to CONSORT for Cluster RandomizedTrials	Page 2-3, line 88-96. Page 3, line 99-105
	3b	Important changes to methods after trial commencement (such as eligibilitycriteria), with reasons		No changes
Participants	4a	Eligibility criteria for participants	When applicable, eligibility criteria for settings and those delivering the interventions	Page 3, line 122-123, line 131-133
	4b	Settings and locations where the data were collected		Page 3, line 120-122
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they are actually administered		Page 3, line 108-118
	5a		Extent to which interventions were actually delivered by providers and taken up by participants as planned	

	5b		Where other informational materials about delivering the intervention can be accessed	Amasene et al. (2019) (doi:10.3390/nu11102337)
	5c		When applicable, how intervention providers were assigned to each group	Not applicable
Outcomes	6a	Completely defined pre-specified outcomes, including how and whenthey were assessed		Page 3-4, line 135-185
	6b	Any changes to trial outcomes after thetrial commenced, with reasons		No changes
Sample Size	7a	How sample size was determined		Page 4, line 187-191
	7b	When applicable, explanation of any interim analyses and stopping guidelines		Not applicable
RANDOMISATION				
Sequence generation	8a	Method used to generate the random allocation sequence		Page 3, line 102-105
	8b	Type of randomisation; detail of any restriction (such as blocking and blocksize)		Page 3, line 102-105
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence, describingany steps taken to conceal the sequence until interventions were assigned		Page 3, line 102-105
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions§		Page 3, 103-105
Awareness of assignment	11a	Who was aware of intervention assignment after allocation (for example, participants, providers, thoseassessing outcomes), and how any masking was done		Page 3, 103-105

	11b	If relevant, description of the similarityof interventions		
Analytical methods	12a	Statistical methods used to comparegroup outcomes	How missing data were handled,with details of any imputation method	Page 4-5, line 192-215
	12b	Methods for additional analyses, such as subgroup analyses, adjusted analyses, and process evaluations		Page 4-5, line 192-215
RESULTS				
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers randomly assigned, receiving the intended intervention, and analysed for the outcomes	Where possible, the number approached, screened, and eligibleprior to random assignment, with reasons for non-enrolment	Figure S1. (Flow-diagram)
	13b	For each group, losses and exclusionsafter randomisation, together with reasons		Figure S1. (Flow-diagram)
Recruitment	14a	Dates defining the periods of recruitment and follow-up		Page 2, line 92
	14b	Why the trial ended or was stopped		
Baseline data	15	A table showing baseline characteristics for each group	Include socioeconomic variableswhere applicable	Page 5
Numbers analysed	16	For each group, number included in each analysis and whether the analysis was by original assigned groups		Page 7
Outcomes and estimation	17a	For each outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	Indicate availability of trial data	Page 5-8, line 219-252
	17b	For binary outcomes, the presentationof both absolute and relative effect sizes is recommended		
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses, adjusted analyses, and process evaluations, distinguishing pre-specified from exploratory		Page 5, 204-215. Page 8 (Table 3) Figure S2.

Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for Harms)		
DISCUSSION				
Limitations	20	Summarize the main results (including an overview of concepts, themes, and types of evidence available), link to the review questions and objectives, and consider the relevance to key groups.	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	Page 8-9, line 258-335 Page 9-10, line 337-362
Generalisability	21	Discuss the limitations of the scoping review process.	Generalisability (external validity, applicability) of the trial findings	Page 9-10, line 337-362
Interpretation	22	Provide a general interpretation of the results with respect to the review questions and objectives, as well as potential implications and/or next steps.	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	Page 10, line 364-381
IMPORTANT INFORMATION				
Registration	23	Registration number and name of trial registry		Page 2, line 90-91
Protocol	24	Where the full trial protocol can be accessed, if available		
Declaration of Interests	25	Sources of funding and other support; role of funders	Declaration of any other potential interests	Page 11, line 396-400 Page 11, line 413-415
Stakeholder investments	26a		Any involvement of the intervention developer in the design, conduct, analysis, or reporting of the trial	Page 10, line 387-395
	26b		Other stakeholder involvement in trial design, conduct, or analyses	No
	26c		Incentives offered as part of the trial	No incentives offered

This table lists items from the CONSORT 2010 checklist (with some modifications for social and psychological intervention trials) and additional items in the CONSORT-SPI 2018 extension. Empty rows in the 'CONSORT-SPI 2018' column indicate that there is no extension to the CONSORT 2010 item

*We strongly recommended that the CONSORT-SPI 2018 Explanation and Elaboration (E&E) document be reviewed when using the CONSORT-SPI 2018 checklist for important clarifications on each item

§An extension item for cluster trials exists for this CONSORT 2010 item

Citations

Montgomery, P., Grant, S., Mayo-Wilson, E., Macdonald, G., Michie, S., Hopewell, S., & Moher, D. (2018). Reporting randomised trials of social and psychological interventions: the CONSORT-SPI 2018 Extension. *Trials*, *19*(1), 407.

Grant, S., Mayo-Wilson, E., Montgomery, P., Macdonald, G., Michie, S., Hopewell, S., & Moher, D. (2018). CONSORT-SPI 2018 Explanation and Elaboration: guidance for reporting social and psychological intervention trials. *Trials*, *19*(1), 406.