UNIVERSIDAD POLITÉCNICA DE MADRID

FACULTAD DE CIENCIAS DE LA ACTIVIDAD FÍSICA Y DEL DEPORTE (INEF)

**The impact of physical activity and fitness on
health biomarkers and oxidative stress in adults
aged over 55 years**

**Impacto de la actividad y la condición física sobre
biomarcadores de salud y estrés oxidativo en
adultos mayores de 55 años**

**International PhD Thesis
Tesis Doctoral Internacional**

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Licenciada en Ciencias de la Actividad Física y del Deporte

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**A quienes me dieron alas para volar
To them who gave me wings to fly**

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En Madrid, 04 de Septiembre de 2017

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List of Abbreviations and Symbols

a.m.	Ante meridiem (= in the morning)
ADLs	Activities of daily living
AI	Adequate intake
AHS	Active and high sedentarism
ALS	Active and low sedentarism
ANOVA	Analysis of variance
β	Standardized coefficients
BMI	Body Mass Index
cm	Centimetre(s)
CAT	Catalase
CDI	Competitive displacement immunoassay
CE	Capillary electrophoresis
CI	Confidence interval
CLIA	Chemiluminescent immunometric assay
CPB	Competitive protein binding
cpm	Counts per minute
CV	Coefficient of variation
CVD	Cardiovascular disease
DACH	German, Austrian and Swiss nutrition societies
DCSP	Dual count solid phase no boil-assay
dL	Decilitre
EAR	Estimated average requirements
EE	Energy expenditure
EI	Energy intake
EIA	Enzyme immunoassays
EDTA	Ethylene-Diamineteaacetic Acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	Retinol Equivalents
et al.	<i>et alii</i> (= and others)
EUROFIT	Council of Europe Committee for the Development of Sport Test Battery
EXERNET	Estudio multi-céntrico para la Evaluación de los Niveles de Condición Física y su relación con estilos de vida saludables en población mayor Española no institucionalizada
ExQ	Cuestionario EXERNET
FE	Iron (serum)
FER	Ferritin
FFM	Fat free mass
FM	Fat mass
FPIA	Fluorescence polarization immunoassay
g	Gram(s)
GC-MS	Gas chromatography- mass spectroscopy
GPx	Glutathione peroxidase
GRd	Glutathione reductase
h	Hour(s)
HC	Hip circumference
HDL-c	High density lipoprotein cholesterol
HoloTC	Holotranscobalamin

HPLC	High performance liquid chromatography
i.e.	id est (=that is)
IAC	Ion capture assay
IHS	Inactive and high sedentarism
ILA	Immunoligand assay
ILS	Inactive and low sedentarism
INE	National Statistics Institute (Spain)
ISAK	International Society for Advancement of Kinanthropometry
Kcal	Kilocalorie(s)
kg	Kilogram(s)
L	Litre(s)
LC-MS	Liquid chromatography- mass spectroscopy
LDL-c	Low density lipoprotein cholesterol
m	Meter(s)
m ²	Square meter(s)
MBA	Microbiological assay
MDA	Malondialdehyde
MEDLINE	U.S. National Library of Medicine®
MEIA	Enzymmunoassay microparticles
METs	Metabolic equivalents
mg	Milligram(s)
min	Minute(s)
mL	Millilitre(s)
MLTPAQ	Minnesota Leisure Time Physical Questionnaire
MMA	Methylmalonic acid
mmHg	Millimetre(s) of mercury
mmol	Millimole(s)
mOsm	Miliosmol
MUFA	Mono-unsaturated fatty acids
MVPA	Moderate to vigorous physical activity
MPO	Myeloperoxidase
n	Number of
ng	Nanogram
nmol	Nanomole
PA	Physical activity
PF	Physical fitness
pg	Picogram
pmol	Picomole
PUFA	Polyunsaturated fatty acids
RA	Radiodilution assay
RBC folate	Red blood cell folate
RIA	Radioimmunoassay
ROS	Reactive Oxygen Species
R ²	Coefficient of determination
s	Seconds
SD	Standard deviation
SFA	Saturated fatty acids
sFolate	Serum folate
SOD	Superoxide dismutase
SPSS	Statistical Package for Social Sciences

TC	Total cholesterol
TG	Triglycerides
tHcy	Total Homocysteine
TPA	Time performing regular physical activity
TRFIA	Time-resolved fluoroimmunoassay
TSS	Time spent sitting
TWH	Time spent walking and time doing housework
UK	United Kingdom
USA	United States of America
VO _{2max}	Maximal oxygen Uptake
vs	Versus
WC	Waist circumference
WHO	World Health Organization
WHtR	Waist to height ratio
yr	Years
α	Alpha
€	Euros
%	Percentage
®	Registered Trademark
°C	Degrees Celsius
µg	Microgram(s)
µmol	Micromole(s)

List of articles from the thesis

1. Aparicio-Ugarriza R, Palacios G, Alder M, Gonzalez-Gross M. *A review of the cut-off points for the diagnosis of vitamin B₁₂ deficiency in the general population*. Clin Chem Lab Med. 2015;53(8):1149-59JC. doi: 10.1515/cclm-2014-0784 (JCR: 3.017).
2. Aparicio-Ugarriza R, Pedrero-Chamizo R, Bibiloni MM, Palacios G, Sureda A, Meléndez A, Tur JA, González-Gross M. *A novel physical activity and sedentary behaviour classification and its relationship with physical fitness in Spanish older adults. The PHYSMED study*. J Phys Act Health. 2017;May 30:1-27. doi: 10.1123/jpah.2016-0263. (JCR: 1.884).
3. Aparicio-Ugarriza R, Luzardo-Socorro R, Palacios G, Bibiloni MM, Julibert A, Tur JA, González-Gross M. *Impact of physical activity and sedentarism on hydration status and liquid intake in Spanish older adults. The PHYSMED study*. Nutr Hosp. 2016; 33(Suppl 3):309. doi: <http://dx.doi.org/10.20960/nh.309>. (JCR: 1.497).
4. Aparicio-Ugarriza R, Luzardo-Socorro R, Palacios G, Bibiloni MM, Argelich E, Tur JA, González-Gross M. *What is the relationship between physical fitness level and macro and micronutrient intake in Spanish older adults?* Eur J Nutr (submitted). (JCR: 4.370).
5. Aparicio-Ugarriza R, Pedrero-Chamizo R, Palacios G, Bibiloni MM, Sureda A, Tur JA, González-Gross M. *Different associations between physical fitness and activity and sedentarism with oxidative stress parameters depending on sex among Spanish older adults*. Exp Gerontol (submitted). (JCR: 3.350).
6. Aparicio-Ugarriza R, Díaz E, Palacios G, Bibiloni MM, Julibert A, Tur JA, González-Gross M. *Which common blood markers could be used to define physical fitness status in older adults?* J Clin Lipidol (submitted). (JCR: 5.812).
7. Aparicio-Ugarriza R, Díaz E, Barrios L, Bibiloni MM, Julibert A, Palacios G, Tur JA, González-Gross M. *An innovative classification of physical fitness and blood markers and their association with socioeconomic status, body composition and quality of life among Spanish old adults. The PHYSMED Study*. J Sci Med Sport (submitted). (JCR: 3.857).

Granted Research Projects

The present PhD Thesis is primarily based on data from the PHYSMED study;

The PHYSMED study is a cross-sectional study on physical activity, physical fitness and sedentarism evaluation and its relation with cardiovascular risk factors among non-institutionalized older adults (Acronym: PHYSMED; project number: PI11/01791) funded by Spanish Ministry of Economy and Competitiveness-Instituto de Salud Carlos III and Physiopathology of Obesity and Nutrition (CIBEROBN) under Grant (CB12/03/30038).

Coordinator: Prof. Dr. Josep Antoni Tur Mari.

Principal investigator for Madrid: Prof. Dr. Marcela González-Gross.

Implication of the PhD candidate: The PhD candidate was involved in the field work in Madrid. The PhD candidate has been processing data and working on the data analysis. Finally, the PhD candidate has been writing the scientific papers.

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- Predoctoral Grant from the ImFine Research Group of the Universidad Politécnica de Madrid. 2014-2015.
- Grant from the Social Council of the Universidad Politécnica de Madrid for internships abroad:
 - Internship in the Nutritional Epidemiology Group, Section of Nutrition and Metabolism, at the International Agency for Research on Cancer (IARC), World Health Organization, Lyon (France). 9 May-19 August 2016 (3 months and 10 days).
- Grant from the ImFine Research Group of the Universidad Politécnica de Madrid to realize internship abroad:
 - Northern Ireland Centre for Food & Health (NICHE), University of Ulster, United Kingdom. June-July 2015 (3 weeks).

Structure of the Thesis

This thesis consists of 6 sections. Section 1 is a general introduction of the whole thesis. Section 2 is corresponded to the objectives. Section 3 achieves the general material and methods explanation of the thesis, Section 4 is the core of the thesis, following the format used by the scientific journals (introduction, material and methods, results, discussion and conclusions). Within this section, each article published and/or submitted is presented (Figure 1). In Article 1, a state of the art with a review of vitamin B₁₂ and its co-markers cut-off points is presented. Article 2 contains a classification of physical activity and sedentary behaviour and the association with physical fitness. Article 3 includes a description of hydration status according to physical activity and sedentary behaviour classification, and physical fitness level. Article 4 analyses the relationship between physical fitness and nutrient intake. In Article 5, oxidative stress parameters will be analysed which could health markers could have an effect. Article 6 studies the association between different biomarkers and physical fitness. In Article 7, an overall of physical fitness and blood markers classification and the link with socioeconomic status, body composition, health-related status and physical activity and sedentary behaviour will be taken into account. Section 5 contains a general discussion of the entire thesis and summarises the main outcomes of the thesis studies. Lastly, section 6 includes the specific and general conclusions of the thesis. Therefore, there have been some repetitions amongst articles as well. For the reader's benefit, references of each article are removed and placed at the end of the thesis.

Figure 1. Description of the main variables studied in each article.

ABSTRACT

The current prevalence and the predicted increase of older population is a key health challenge in our society. Regular physical activity provides significant health benefits and low physical fitness is a stronger predictor of cardiovascular and all-cause mortality than other risk factors. A sedentary behaviour has a negative impact on health. Nutrient status affects risk-factor levels during life and may have an even greater impact in older adults. Furthermore, biomarkers are a useful tool to measure the effect of exercise. It is essential to get deeper into the association between biomarkers and physical fitness level, and other health determinants. However, there is an increase of non-communicable diseases being, a difficult task to provide effective policies and interventions. An inadequate classification of people according to physical activity and sedentary behaviours could be one the causes.

The main purposes of the thesis were (1) to examine the associations between physical fitness, physical activity, sedentary behaviour, and several environmental and biochemical variables in Spanish older adults. These variables include, among others, nutritional status, health-related quality of life, vitamins and oxidative stress parameters; (2) to clarify the difficulty to categorize people and provide more reliable data. Four different classifications have been suggested in the current thesis: (i) Combining physical activity and sedentary behaviours, (ii) According to physical fitness, (iii) Considering reference ranges from each blood marker, (iv) Combining physical fitness and blood markers, called Total Index.

The current thesis is based on a cross-sectional multicentre study collecting data on 433 Spanish non-institutionalized older adults (57% females, aged over 55 yr) from Madrid and Balearic Islands regions. Each classification was in turn divided into different groups as presented below:

- Physical activity and sedentary behaviour classification: inactive and high sedentarism, inactive and low sedentarism, active and high sedentarism and, active and low sedentarism according to validated questionnaires.
- Physical fitness classification: low, medium and high physical fitness level according to four validate physical fitness test.
- Blood markers: each biomarker was categorized as being within the reference range and out of the reference range considering as reference range those established by laboratories.

- Total index (physical fitness & blood markers classifications): Scores of each biomarker were added together and the total score was divided into tertiles. After that, the three levels of physical fitness and three groups of biomarkers were combined together and a total index with nine different subgroups was created.

The main outcomes of this thesis are: a) Males spent more time doing regular physical activity but less time walking and working at home than females. b) The proposed classification combining physical activity and sedentary behaviour is valid for males as it discriminates according to their physical fitness. c) Active and low sedentary participants (both males and females) obtained better results in the physical fitness tests. d) Participants in the active and low sedentary behaviour group consumed higher amounts of water and other beverages than the others groups; however, different patterns were observed according to their physical fitness level. e) Physical fitness level is related to nutrient intake, presenting greater relationships with micronutrient intake than macronutrient intake. f) There was a high inadequate intake of potassium, vitamin D and vitamin E below requirements, which was present in almost 85% of older adults, independently of their physical fitness level. g) Sex was identified as the main factor that had an effect on oxidative stress parameters. h) High lipid peroxidation measured through malondialdehyde was found in the high physical fitness group in females, whereas higher antioxidant enzyme activity measured by superoxide dismutase was observed in the high physical fitness group in males. i) There was a considerable percentage of participants who showed values out of reference range for vitamin 25[OH]D (68%) and total cholesterol (59 %), independently of physical fitness. j) More abnormal values were observed for total homocysteine, creatinine, total cholesterol, high density lipoprotein cholesterol and low density lipoprotein cholesterol in the low physical fitness group; meanwhile, less abnormal values were found for vitamin B₁₂ and triglycerides in the low physical fitness group. k) Physical fitness index and blood index were independent between them; thus, each health marker should be measured in an independent way. l) Total index (physical fitness & blood markers) was associated with socioeconomic status, physical activity and sedentary behaviour, abdominal obesity and health-related quality of life. (m) In the model, the main predictor of the total index was education. Likewise, participants with primary school education, in the active and low sedentary group, also were categorized with less abdominal obesity.

The holistic and novel approach can contribute to establish public health policies and new interventions in older adults.

Key words: physical activity; physical fitness; sedentary lifestyle; biomarkers; oxidative stress; nutritional status; middle aged; elderly.

Table 1. Summary of state of the art and what this Thesis adds.

No article	What is already known on this topic?	What does this PhD Thesis add?
1	There is no agreement on the cut-off points for vitamin B ₁₂ and its co-markers, such as folate, holotranscobalamin, methylmalonic acid and homocysteine. Furthermore, vitamin B ₁₂ and folate deficiency is still an important nutritional problem worldwide as subclinical deficiency affects well defined risk groups such as older subjects.	In a review, inconsistency due to a broad range of cut-off points for vitamin B ₁₂ and its biomarkers were identified. We proposed that it is necessary to establish different reference cut-offs according to age, sex, fortified food consumption considering the analytical methods used.
2	Active people manifest lower rates of all cause-mortality, but sedentary behaviour has emerged as a risk factor of early mortality. Older adults are commonly classified as physically active or inactive without considering time spent on sedentary behaviour. Noteworthy, people can achieve high levels of physical activity and also spend many hours sitting.	The proposed classification is valid as it classifies participants according to their physical activity and sedentary behaviour, and outcomes are related to physical fitness, concretely in males. Moreover, this innovative classification offers a practical approach that could be used by health authorities, researchers and physicians.
3	Hydration status is an emerging topic in nutritional epidemiologic studies; however, data on fluid and water intake are scarce, especially in older adults. Likewise, individual's factors such as physical activity, physical fitness or sedentary behaviours could affect the total fluid and water intake.	To offer, for the first time, water and fluid intake data taking into account physical fitness, and physical activity and sedentary behaviour through the proposed classification. Both inactive groups consumed less total liquid than the active and low sedentary group. However, different patterns of fluid intake were observed according to physical fitness level.
4	Physical fitness is in part genetically determined, and it is also influenced by environmental factors, principally physical activity; nevertheless, it is not well-known how it is related to diet. Additionally, there are few studies that have studied the relationship between physical fitness with macro and micronutrient intake, especially in Spanish older population.	Physical fitness has been related to nutrient intake. Significant associations were observed between several micronutrients and physical fitness level. In fact, participants in the high physical fitness group showed better micronutrient intake profile than the other groups. Nonetheless, a considerable inadequate intake below requirements of several micronutrients was observed, independently of physical fitness. Thus, this data suggest contemplating supplementation for micronutrients at risks.

5	<p>Aging is associated with an oxidative imbalance and with advancing age, antioxidant enzymes diminish their activity. Genetic and environmental factors and lifestyles behaviours could induce abnormal increase in free radical production and/or decrease in antioxidant defence. Nevertheless, available data concerning influence on physical activity and physical fitness and oxidative stress is still scarce.</p>	<p>Our data indicate that several oxidative stress parameters are related to lifestyles. Sex has been identified as influential factor. Indeed, malondialdehyde showed a negative relationship with physical fitness level in females, whereas superoxide dismutase displayed a positive association with physical fitness level in males.</p>
6	<p>Biomarkers can provide important insights related to the impact of exercise on different systems, organs and tissues. Decreases in physical fitness level are associated with several negative health effects. It remains an important goal to get deeper into the understanding of the relationship between biomarkers and physical fitness. Besides, there are not studies investigating the association between abnormal/normal biomarkers values and physical fitness level.</p>	<p>This thesis analyses numerous biomarkers such as vitamin B₁₂, folates, total homocysteine and vitamin D, among others, according to their physical fitness level. Biomarkers were categorized as being within reference range or out of the reference range. Surprisingly, lower abnormal values for vitamin B₁₂ and triglycerides were observed in the low physical fitness group, although higher abnormal values were obtained for lipid profile (except triglycerides), total homocysteine and creatinine in the low physical fitness group than in the high physical fitness group. The holistic approach used can contribute to a better understanding of the association between biomarkers and physical fitness level.</p>
7	<p>Older population is increasing quickly and life expectancy is clearly rising. The socioeconomic and physical environment and lifestyle are determinants of good health and quality of life. But there are no studies that have examined whether Spanish groups of older adults have identifiable ways of patterning their physical fitness and blood markers across different domains.</p>	<p>We present a novel combined classification of physical fitness and blood markers. Through this classification, characterized patterns considering their socioeconomic status, body composition and health-related status were provided. The main predictor variable was education in the model. Waist-to-height ratio, physical activity and sedentary behaviour should be considered. Also, current income, body mass index, anxiety, depression and pain should be included in the studies.</p>

RESUMEN

La población de edad avanzada supone un desafío clave de salud dada la prevalencia actual y el aumento previsto de la misma. La práctica de actividad física regular proporciona beneficios significativos para la salud, siendo el fitness un fuerte predictor cardiovascular y de todas las causas de mortalidad en comparación con otros factores de riesgo. Por el contrario, el comportamiento sedentario tiene un impacto negativo sobre la salud. La nutrición influye sobre los factores de riesgo durante toda la vida, provocando un mayor impacto en los adultos. Además, los biomarcadores son una herramienta útil para medir el efecto del ejercicio siendo esencial profundizar en la asociación entre biomarcadores y el nivel de fitness junto con otros factores determinantes de salud. Sin embargo, hay un aumento de las enfermedades no transmisibles siendo una tarea difícil proporcionar políticas e intervenciones eficaces. La inadecuada clasificación de las personas según las recomendaciones de actividad física y sedentarismo podría ser una de las causas.

Los principales objetivos de esta tesis fueron (1) examinar las asociaciones entre el fitness, la actividad física, el comportamiento sedentario, y diversas variables ambientales y bioquímicas en adultos mayores españoles. Estas variables incluyen estado nutricional, calidad de vida relacionada con la salud, vitaminas y parámetros de estrés oxidativo, entre otros; (2) Aclarar la dificultad de clasificar a las personas y proporcionar datos más fiables. Se han propuesto cuatro clasificaciones diferentes en la presente tesis: (i) De acuerdo al nivel de actividad física y el tiempo sedentario, (ii) De acuerdo al nivel de fitness, (iii) Considerando los rangos de referencia de cada marcador de sanguíneo, (iv) combinando la clasificación de fitness y la clasificación de los marcadores sanguíneos, titulado Índice Total.

La presente memoria de Tesis Doctoral está basada en un estudio multicéntrico transversal en el que se han analizado un total de 433 adultos españoles no institucionalizados mayores de 55 años (57% mujeres), procedentes de las Comunidades de Madrid e Islas Baleares.

Cada clasificación se dividió a su vez en los siguientes grupos:

- Clasificación de actividad física y sedentarismo: inactivo y alto sedentarismo, inactivo y bajo sedentarismo, activo y alto sedentarismo y, activo y bajo sedentarismo de acuerdo a cuestionarios validados.
- Clasificación de fitness: bajo, medio y alto nivel de fitness de acuerdo a cuatro test validados de condición física.

- Marcadores sanguíneos: cada biomarcador se categorizó estando dentro del rango de referencia y fuera del rango de referencia considerando los rangos de referencia establecidos por los laboratorios.

- Índice Total (clasificación de fitness y marcadores sanguíneos): Cada biomarcador se puntuó y la puntuación total se dividió en terciles. Después de ellos, se combinaron los tres niveles de fitness y tres grupos de biomarcadores creándose un Índice Total con nueve subgrupos diferentes.

Los principales resultados de esta tesis son los siguientes: a) Los varones realizaron más actividad física regular pero dedicaron menos tiempo a caminar y a las tareas del hogar que las mujeres. b) La clasificación propuesta, combinando actividad física y comportamiento sedentario es válida en los hombres, ya que discrimina de acuerdo a su fitness. c) El grupo de participantes activos y de bajo comportamiento sedentario obtuvo mejores resultados en las pruebas de condición física en ambos sexos. d) Los participantes del grupo activo y bajo comportamiento sedentario bebieron mayores cantidades de agua y otras bebidas; sin embargo, se encontraron diferentes patrones de ingesta de agua y bebidas de acuerdo a su nivel de fitness. e) El nivel de fitness está relacionado con la ingesta de nutrientes, presentando mayores relaciones con la ingesta de micronutrientes que con la ingesta de macronutrientes. f) Más del 85% de los adultos mayores mostró una ingesta inadecuada por debajo de los requerimientos de potasio, vitamina D y vitamina E, independientemente de su nivel de fitness. g) El sexo fue identificado como el principal factor que tiene efecto sobre los parámetros de estrés oxidativo. h) Se observó una mayor peroxidación lipídica medida a través del malondialdehído en el grupo de alto fitness en mujeres, mientras que se mostró una mayor actividad enzimática antioxidante medida por la superóxido dismutasa en el grupo de alto fitness en hombres. i) Un alto porcentaje de participantes mostraron valores sanguíneos fuera de rango, especialmente de la vitamina D (68%) y el colesterol total (59%), independientemente del fitness. j) Se observaron mayores niveles fuera de rango para la homocisteína total, creatinina, colesterol total, lipoproteína de alta densidad y lipoproteína de baja densidad en el grupo de bajo nivel de fitness; mientras que menores niveles fuera de rango en la vitamina B₁₂ y triglicéridos fueron encontrados en el grupo de bajo fitness. k) Los índices de fitness y marcadores sanguíneos son independientes entre ellos; por tanto, cada marcador de salud debe ser medido de forma independiente. l) Se encontró una asociación entre Índice Total y el estado socioeconómico, actividad física y comportamiento sedentario, obesidad

abdominal y parámetros de salud. (m) En el modelo, la principal variable predictora del Índice Total fue la educación. Así mismo, los participantes con estudios primarios, en el grupo de activos y bajo sedentarismo, también fueron categorizados con una menor obesidad abdominal.

Este novedoso enfoque holístico puede contribuir a establecer políticas de salud pública y nuevas intervenciones en población mayor.

Palabras Clave: actividad física; condición física; estilo de vida sedentario; biomarcadores; estrés oxidativo; estado nutricional; personas mediana edad; personas mayores.

Table 2. Sumario sobre el estado de arte y los nuevos datos que añade la Tesis Doctoral.

Nº artículo	¿Qué se sabe en este ámbito?	¿Qué añade esta Tesis Doctoral?
1	Existe falta de consenso acerca de los puntos de corte para la vitamina B ₁₂ y marcadores relacionados, tales como folato, holotranscobalamina, ácido metilmalónico y homocisteína. Además, la deficiencia de vitamina B ₁₂ y folato es todavía un problema nutricional importante a nivel mundial. La deficiencia subclínica afecta a grupos de riesgo bien definidos, entre ellos, las personas mayores.	En una revisión, se identificaron inconsistencias debido a los amplios rangos de puntos de corte para la vitamina B ₁₂ y sus marcadores relacionados. Nosotros proponemos que es necesario establecer diferentes puntos de corte de acuerdo a la edad, sexo, consumo de alimentos enriquecidos y considerando la técnica de análisis de los biomarcadores estudiados.
2	Las personas activas manifiestan tasas más bajas de mortalidad por todas las causas. El comportamiento sedentario ha emergido como un factor de riesgo de mortalidad temprana. Las personas mayores son comúnmente clasificadas por su nivel de actividad e inactividad física sin considerar el tiempo de comportamiento sedentario. Cabe destacar que las personas pueden alcanzar niveles altos de actividad física y también, estar muchas horas sentadas.	La clasificación propuesta es válida para clasificar a los participantes de acuerdo a su nivel de actividad física y tiempo sentado. La clasificación se relaciona con el fitness, especialmente en hombres. También, esta innovadora clasificación ofrece un enfoque práctico que podría ser utilizado por las autoridades de salud, investigadores y médicos.
3	La hidratación es un área emergente en estudios nutricionales epidemiológicos; sin embargo, los datos sobre ingesta de líquidos y agua son escasos, especialmente en personas mayores. De igual manera, los factores individuales como la actividad física, fitness o comportamiento sedentario podrían afectar la ingesta total de líquidos y agua.	Ofrecer, por primera vez, la ingesta total de agua y líquidos teniendo en cuenta el fitness y la clasificación propuesta sobre actividad física y sedentarismo. Ambos grupos inactivos consumieron menores cantidades de líquido total en comparación con el grupo activo y bajo sedentarismo. Sin embargo, diferentes patrones de ingesta de líquidos fueron encontrados de acuerdo al nivel de fitness.
4	El fitness está determinado en parte por la genética, y también influenciado por factores medioambientales, principalmente la actividad física. No obstante, no se sabe	El fitness está relacionado con la ingesta de nutrientes. Se observaron mayores asociaciones entre varios micronutrientes y el nivel de fitness. De hecho, los

	<p>bien como el fitness se relaciona con la dieta. Además, hay pocos estudios que hayan analizado la asociación entre el fitness y la ingesta de macro y micronutrientes, especialmente en población española adulta.</p>	<p>participantes del grupo de alto fitness mostraron un mejor perfil de ingesta de micronutrientes en comparación con el resto de grupos. Pese a ello, se observó una ingesta inadecuada considerable por debajo de los requerimientos para varios micronutrientes, independientemente del nivel de fitness. Por tanto, estos datos sugieren la necesidad de suplementar para aquellos micronutrientes en riesgo.</p>
5	<p>El envejecimiento está asociado con un desequilibrio oxidativo y al avanzar la edad, las enzimas antioxidantes disminuyen su actividad. Los factores genéticos, los comportamientos ambientales y de estilos de vida podrían inducir un aumento anormal de la producción de radicales libres y/o la disminución de la defensa antioxidante. Sin embargo, los datos disponibles sobre el efecto de la actividad física y el fitness sobre el estrés oxidativo todavía son escasos.</p>	<p>Nuestros datos indican que varios parámetros de estrés oxidativo están relacionados con el estilo de vida. El sexo ha sido identificado como un factor influyente. De hecho, el malondialdehído mostró una relación negativa con el fitness en las mujeres, mientras que la superóxido dismutasa mostró una asociación positiva con el nivel de fitness en los hombres.</p>
6	<p>Los biomarcadores pueden proporcionar información importante relacionada con el impacto del ejercicio en diferentes sistemas, órganos y tejidos. Igualmente, la disminución en el nivel de fitness está asociada con efectos negativos para la salud. Continúa siendo un objetivo importante profundizar en la relación entre los biomarcadores y el fitness. Además, no hay estudios que investiguen la asociación entre valores anormales/normales de biomarcadores y el nivel de fitness en mayores.</p>	<p>Esta tesis analiza numerosos biomarcadores como vitamina B₁₂, folatos, homocisteína total y vitamina D, entre otros, según el nivel de fitness de los participantes. Los biomarcadores fueron categorizados dentro y fuera de su rango de referencia. Sorprendentemente, menores valores fuera de rango fueron observados para la vitamina B₁₂ y los triglicéridos en el grupo de bajo fitness. Mayores niveles fuera de rango se encontraron para el perfil lipídico (excepto los triglicéridos), la homocisteína y la creatinina en el grupo de bajo fitness en comparación con el grupo de alto fitness. El enfoque holístico utilizado puede contribuir a una mejor comprensión de la relación entre los biomarcadores y el nivel de fitness.</p>

7	<p>La población de edad avanzada y la esperanza de vida están aumentando rápidamente. El entorno socioeconómico, físico y el estilo de vida son determinantes de la buena salud y calidad de vida. Sin embargo, no existen estudios que hayan categorizado a personas mayores españolas en función del fitness y marcadores sanguíneos en diferentes dominios.</p>	<p>Nosotros presentamos una clasificación novedosa y combinada de fitness y marcadores sanguíneos. A través de esta clasificación, se caracterizaron patrones de comportamiento considerando el estado socioeconómico, composición corporal y calidad de vida. La educación fue la principal variable predictora del modelo. El ratio cintura-altura, la actividad física y el comportamiento sedentario deberían ser considerados. Asimismo, la renta, el índice de masa corporal, la ansiedad, la depresión y el dolor deberían ser incluidos en este tipo de estudios.</p>
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1 INTRODUCTION

1.1 Research Background

Aging is a natural and multi-factorial phenomenon involving complex interactions between biological and molecular pathways (179,326,360,362). The aging process and the development of chronic diseases are accompanied by increase deoxyribonucleic acid damage, epigenetic modifications, and telomere shortening (145,364). The production of reactive oxygen species by mitochondria accumulated over the lifespan leads to a state of chronic oxidative stress with advancing age (264).

Older adults are increasing quickly and Spain is no an exception. Spanish aging population grows more than 18% by 2016, and rapidly will increase to 38.7% by 2061 (1). The European Union as a whole is also confronted with an ageing population and the latest data established that the number of people aged over 65 yr was 18.5% in 2015 (338). A further projection would give a percentage of almost 30% by 2080 (338).

An increase in the age of older adults is accompanied by several physiological issues, as decline in exercise capacity, loss of muscle strength and power, lower lung capacity, less walking and ability to perform activities of daily living (ADLs) (230,288). Furthermore, aging is associated with an increased risk of chronic conditions and diseases such as cognitive impairment, cardiovascular disease (CVD), cancers and metabolic syndrome (47). Due to prolonged life expectancy, age-related diseases have increased in worrying proportions in recent decades (69).

In this sense, the World Health Organization (WHO) has defined the term “healthy aging” as the process of developing and maintaining the functional ability that enables wellbeing in older age (377). The role of physical activity (PA) and dietary habits in decreasing the development of chronic disease is becoming increasingly important (126) and both aspects can contribute to have a healthy aging.

It is well-known that regular PA can provide significant health benefits to older adults population (131,346) and PA can prolong yr of ADLs (244,340), decrease disability (208) and improve the quality of live (88). PA is defined as any bodily movement produced by skeletal muscles that derive in energy expenditure while physical exercise is a subset of physical activity that is structured, repetitive, planned and has the objective of maintaining or improving PF (64,256). Measuring PA levels to assess the physical condition of an older person and, therefore, their ability to carry out everyday activities in an independent manner cannot be the most accurate method (65,98,312).

Indeed, physical fitness (PF) has been postulated as marker of current and future health (43,256,269,362), and PF is a stronger predictor of cardiovascular and all-cause mortality than other risk factors (even PA). PF is the capacity to perform physical activity and join in the body functions (cardio-respiratory, skeleto-muscular, hemato-circulatory and endocrine-metabolic), and an extensive range of physiological and psychological qualities (256). Moreover, PF is partly genetically determined, although it can also be highly influenced by environmental factors being physical exercise one of the main determinants (256). PF is often measured by means of objective tests of functional capacity (85) and can be analysed by each individual capacity or by grouping them up in a score (271). Aerobic capacity, muscular endurance, muscle strength and balance are the most linked components of PF (13,269,345). Flexibility, speed of the movement and reaction time also take part of the PF (47).

From this point of view, PF tests are used for monitoring the biological aging process (362). A slower walking, a weaker grip strength, poorer standing balance performance and, lower extremities strength as less repetition in chair stand test are all related to significantly greater mortality rates, independent of age in older populations (362). Besides, poorer walking speed, balance and handgrip and extremities strength are linked to higher risk for CVD (362), dementia, institutionalization and difficulties doing ADLs (76).

Low aerobic fitness is related to CVD, high blood pressure, diabetes, and obesity (47). The gold standard to measure cardiovascular capacity is by means of maximum oxygen consumption (144). This capacity decreases approximately 10% per decade with advancing age in both sexes in spite of PA level (144). Recently, telomeres have been received attention as anti-aging marker and different studies are analysing the relationship between high PA and PF levels and telomeres (330).

At the level of muscular strength, its highlight to indicate that muscular strength is one of the most determinant capacities in older adults. Lower levels of grip strength are also associated with functional deterioration measured by ADLs self-reported (367) as well as in reducing the risks of falls and fall-related injuries (269). Likewise, aging is related to body composition changes taking into account decreased muscle mass and organ mass and increased body fat as well as changes in the muscle structure and function (89). Meanwhile, the relationship between muscle mass and strength is not linear (138,164). Regarding agility and dynamic balance, both capacities are potentially

predictors of disability and both are related to major risk of falling and fear (84,306), being a high incidence factor at old stage life (238).

Although the benefits of doing regular PA and improving PF are well-recognized, there is an increase of time spent sitting (TSS). Sedentary behaviour is any waking activity characterized by an energy expenditure ≤ 1.5 metabolic equivalents (METs); whereas in a sitting, reclining or lying posture (318,349). A total of 41.3% of the Spanish population (337) and 42% of Europeans aged over 15 yr claims to be sedentary (381). In a representative Spanish population, a total of 27% Spanish adults did not perform any PA (228). Additionally, worthwhile to note is that 54.3% and 21.1% never performs vigorous and moderate PA, respectively (228). TSS has been displayed to be a modifiable risk factor of type 2 diabetes mellitus (90), CVD (176), some cancers, obesity (161) and skeletal diseases (125). Furthermore, TSS has a negative impact on several aspects of mental health, disablement, overall mortality, and frailty as well as low quality of life, independently from PA levels (31). Sedentary behaviours affect muscle physiology, lead to weakness and sarcopenia (125). Recently, it has been suggested that prolonged TSS might encourage visceral or ectopic fat accumulation (146).

Another key point supporting healthy aging is the role of nutrition status. Dietary habits seem to affect risk-factor levels during life and may have an even greater impact in older population (379). However, their nutritional requirements are not well defined considering that basal metabolic rate and lean body mass decrease with advancing age, and also energy requirements per kilogram of body weight is reduced in older adults (379). Adequate diet includes also an adequate micronutrient intake. Spanish and European studies concluded that nutrition in the old adults was inadequate and unbalanced (95). Energy requirements diminish with advancing age, and older adults trend to become less active when they get older (379). Likewise, elderly population is mostly vulnerable to malnutrition and diet-related non-communicable diseases are the most common cause of death worldwide (379). The role that micronutrients also play in promoting health and preventing non-communicable disease is getting considerable consideration.

Hydration status also must be considered within nutrition status. Quantification of water and beverage intake is an emerging topic in nutritional sciences as the optimal functioning of body requires a proper hydration level (121,170). Water consumption is associated with several health benefits (278). Adequate fluid intake is also essential for

optimal urinary, gastrointestinal (278), and cognitive tasks (24); keeping blood glucose homeostasis (71); and possibly decreased mortality risk (220). In older adults, adequate fluid consumption has been connected with fewer falls, fewer rates of constipation and laxative use, as well as better rehabilitation outcomes in orthopaedic patients (225). All the above-mentioned aspects can be measured through biomarkers analyses, which are a useful tool to measure the effect of PA, PF, nutrition status and hydration, among others (258,362). There is no criterion to establish which biomarkers are the best for assessing healthy ageing yet, and this creates several controversies (186). Nutrition-related markers are several, although the majority of the studies have focused on vitamin D, B₁₂, B₉ and B₆ but data are not convincing (362). Indeed, B vitamins complex and total homocysteine have been associated with numerous diseases such as cognitive decline, dementia, fracture, stroke, mortality (81,155,250,314,327,384). Older people are susceptible to develop vitamin D deficiency because of various risk factors: decreased dietary intake, diminished sunlight exposure, reduced skin thickness, impaired intestinal absorption, and impaired hydroxylation in the liver and kidneys (163).

In turn, no single biomarker can provide a valid measure of healthy aging because the multiplicity of the many biological and molecular mechanisms of aging. Likewise, oxidative stress is defined as the lack of balance between the occurrence of reactive oxygen/nitrogen species (ROS/RNS) and the organism's capacity to counteract their action by the antioxidative protection systems (38). Oxidative stress contributes to many pathological conditions, including cancer (353), neurological disorders (169), atherosclerosis, asthma (15), ischemia (87), chronic obstructive pulmonary disease (26), among others (38). In the current thesis, different oxidant and antioxidant markers were measured. The current thesis tries to provide a measure of ongoing optimal health through analysing health markers in Spanish older adults (362).

2 OBJECTIVES

General objectives

-To analyse the relationships between physical fitness and several environmental and biochemical variables in Spanish older adults. These variables include, among others, physical activity, sedentary behaviours, nutritional habits, hydration status, vitamins and oxidative stress parameters.

-To classify participants according to health markers in order to can offer more effective strategies, policies and interventions. This holistic approach will contribute to a better comprehension of which current markers of health will be more useful to advice of a higher risk of disease in Spanish older people.

Specific objectives

The following research aspects will be considered:

Article 1:

- To study the state of the art of vitamin B₁₂ and its co-markers cut-off points used to determine the diagnosis of vitamin B₁₂ deficiency.

Article 2:

- To propose an integrative classification combining physical activity and sedentary behaviour. Then, to evaluate the association between this classification and physical fitness in Spanish older adults.

Article 3:

- To report hydration status in Spanish older population according to physical activity and sedentary behaviour classification and physical fitness level.

Article 4:

- To assess the association between nutrients intake with physical fitness level, identifying older adults at risk of vitamins and minerals deficiency.

Article 5:

- To analyse the association between physical fitness and parameters related to unbalanced cell oxidative equilibrium.

Article 6:

- To evaluate and establish the association between physical fitness levels and blood biomarkers which are related to disease risk in Spanish older adults.

Article 7:

- To create a novel and combined classification of physical fitness and blood markers, providing predictors variables associated with socioeconomic status, body composition and quality of life.

3 GENERAL MATERIAL AND METHODS

This doctoral thesis is based on data obtained from the PHYSMED study:

3.1 The PHYSMED project-study design

The PHYSMED study is a cross-sectional study supported by the Spanish Ministry of the Economy and Competitiveness-Instituto de Salud Carlos III which aimed to evaluate physical activity (PA), physical fitness (PF) and sedentarism behaviour and its relation with cardiovascular risk factors among non-institutionalized old adults (Acronym: PHYSMED; project number: PI11/01791), under coordination of Prof. Josep Antoni Tur Mari (Universidad de las Islas Baleares).

The working group is composed by two research groups belonging to two different research centres (Figure 2):

- Research Group on Nutrition, Exercise and Healthy Lifestyle (ImFine), Department of Health and Sport Performance, Faculty of Physical Activity and Sport Science-INEF, Universidad Politécnica de Madrid, Madrid.
- Research Group on Community Nutrition and Oxidative Stress (NUCOX), Fundamental Biology and Health Sciences, Faculty of Science, University of the Balearic Islands, Mallorca.

Figure 2. Participating centres in the PHYSMED Study.

3.1.1 Study protocol

The long version of the protocol is available in the Appendix 1. Individuals from Madrid who agreed to participate in the study were invited to be present at the Faculty of Physical Activity and Sport Sciences-INEF belonging to the Universidad Politécnica de Madrid and participants from Mallorca at the Faculty of Sciences belonging to the University of Balearic Islands.

The study was carried out in two phases. On the first day, blood samples were collected and afterwards participants responded to the EXERNET questionnaire which aimed to assess general information as well as PA and sedentarism behaviour. In addition, the Minnesota Leisure-time Physical Activity Questionnaire (MLTPAQ) proposed to assess PA during last year. Likewise, the first 24h dietary recall and a semi-quantitative food frequency questionnaire aimed to assess their nutritional habits were evaluated.

On the second day, with a span of at least a week and within a period of 2 or 3 weeks, body composition (anthropometric measurements and bioimpedance analysis), blood pressure measurements as well as PF tests battery were performed. A hydration questionnaire developed by the Research Group and the second 24h dietary recall were evaluated.

Figure 3. Summary protocol of the thesis.

3.1.2 The PHYSMED project- participant recruitment

In this cross-sectional study, non-institutionalized older adults from 2 Regions in Spain: Madrid and Mallorca were evaluated. Following a snowball sampling, the recruitment of participants was carried out through health centres, sport federations, sport facilities and municipal clubs located at Madrid and Mallorca.

Likewise, participants were selected as follows: active (> than 4 h of PA/week, at least on three days), sedentary (< than 2 h of PA/week), and intermediate (not included in one

of the other two groups). Data collection took place from April 2013 to May 2014 in Madrid and Mallorca.

The exclusion criteria were age under 55 yr, being institutionalized, suffering from a physical or mental illness which would have limited their participation in the physical fitness tests or their ability to respond to the questionnaires or drug intake for clinical research.

The sample consisted of 477, however, 25 participants declined the participation and 19 participants did not complete at least half of the tests. After finishing the field work a total of 433 older adults, 186 males and 247 females, finally took part in the study. Figure 4 sums up the study sample in each article of the current thesis.

Figure 4. Description/Diagram of the study sample in each article.

3.2 Ethical issues

The study was performed according to the principles established with the Declaration of Helsinki 1964 and further amendment and other national regulations for research projects involving human participants: Protection of personal data, Law 15/1999 of 13 December on the Protection of Personal Data provided in the current legislation (Royal Decree 1720/2007 of 21 December). The protocol study was approved by the Ethical Committee of the Universidad Politécnica de Madrid. All participants were informed

about the nature and purpose of the study (Appendix 2). Informed consent was signed by all individual participants included in the study (Appendix 3).

3.3 Blood samples processing

3.3.1 Sample pre-treatment and biochemical analysis

In the first visit, a fasting blood analysis (12h) took place between 9 to 10:30 a.m. Approximately, 40 mL of blood was collected from an antecubital vein and the extraction was performed by standard venepuncture on vacuum Vacutainer® tubes with separation gel for serum or EDTA as anticoagulant for analysing the different biomarkers and blood parameters.

For hematological parameters, a complete hematological analysis was performed within the first hour after extraction and was obtained by an automated hematology analyser (ADVIA 120 Siemens Health Care Diagnostics, SA).

Gel for serum tubes were immediately placed on ice and after coagulum formation were centrifuged at 3,000 rpm for 10 minutes. Furthermore, the rest of serum was alicuoted and stored at -80°C until processing.

Routine biochemistry analysis was carried out in fresh serum with the Beckman AU400 analyser (Beckman AU400, Beckman Instruments, Ltd., Bucks, UK) by photometric method, except for creatinine (by colorimetric method), using standard methodologies. Moreover, serum osmolarity was determined by freezing point depression with an Osmo Station OM-6050 (Menarini, Diagnostics, Italy).

Hematology, routine biochemistry and osmolarity were performed in situ at the clinical chemistry laboratories of High Sports Council (Madrid) and Hospital Son Espases & University of Balearic Islands (Mallorca). After a blood sampling a breakfast was offered to each participant.

Analysis of vitamin B₁₂, serum folate, red blood cell folate (from EDTA tubes), vitamin 25[OH]D and total homocysteine were centralized at the High Sports Council Laboratory in Madrid. Therefore, serum samples from Palma de Mallorca were dry-ice shipped to Madrid and analysed together with those serum samples stored in Madrid at -80°C, by electrochemoluminescence method (Elecsys 2010, Roche Diagnostics, Switzerland), except vitamin 25[OH]D which was determined using E411 analyser

(Roche Diagnostics, Switzerland). The method and devices used for the biochemical analysis are presented in Table 3.

3.3.2 Seasonality and latitude

As blood sampling was performed throughout a year, season was considered for further analysis. Autumn-winter samples were coded as 1 and Spring-summer samples were coded as 2. The latitude was also considered in the present thesis. The latitude of each city was obtained from <http://maps.google.es/>. Latitudes of the involved cities were: Palma de Mallorca (39° 34' N) and Madrid (40° 24' N). To make use of this data, latitudes were added to the database as numeric variables with two decimals (i.e. Madrid= 40.24).

3.3.3 Materials

The method and devices used for the blood samples are presented in Table 3.

Table 3. List of methods and devices used for the blood samples.

Parameter	Sample	Method	Analyser
Biomarkers			
Total homocysteine			
Vitamin B ₁₂	Serum		Elecsys 2010,
Serum folate			Roche Diagnostics,
			Switzerland
Red blood cell folate	Erythrocyte hemolysate	Electrochemoluminescence immunoassay	
Vitamin 25[OH]D	Serum		E411 analyser Roche Diagnostics, Switzerland
Osmolarity	Serum	Freezing point depression	Osmo Station OM-6050 (Menarini Diagnosis, Italy)
Lipid profile			
Total Cholesterol			
HDL-Cholesterol			Beckman AU400,
LDL-Cholesterol	Serum	Photometric analyser	Beckman Instruments, Ltd., Bucks, UK
Triglycerides			

Hematological parameters			
Haematocrit			ADVIA 120
Red blood cells	Whole blood	Automated hematology analyser	(Siemens Health Care Diagnostics, SA
Haemoglobin			
Parameter	Sample	Method	Analyser
Biochemistry routine			
Glucose			
Urea			
Uric acid			
Creatinine*			Beckman AU400,
Total protein			Beckman
Albumin	Serum	Photometric analyser	Instruments, Ltd., Bucks, UK.
Iron			
Ferritin			
Glutamic oxalacetic transaminase			
Glutamic-pyruvic transaminase			
Gamma glutamyl transpeptidase			

*Colorimetric method

3.4 Oxidative stress parameters

Oxidative stress parameters were centralized at the University of Balearic Islands and samples were dry-ice shipped from Madrid to Mallorca. Because enzymatic activity measures could only be performed on fresh blood, samples dry-ice shipped from Madrid to Mallorca consisted of erythrocytes only hemolysates and leucocytes concentrates for enzyme concentrations. Therefore, enzyme activity data came only from Mallorca samples.

Superoxide dismutase (SOD) activity was measured in plasma by an adaptation of the method of McCord and Fridovich (221), and determined with a Shimadzu UV-2100 spectrophotometer at 37°C.

Catalase (CAT) activity was measured in neutrophils by the spectrophotometric method of Aebi based on the decomposition of H₂O₂ (3). Myeloperoxidase (MPO) activity of neutrophils was measured by guaiacol oxidation (61). The reaction mixture contained sodium phosphate buffer, pH 7.0 and 13.5 mmol/L of guaiacol. The reaction was initiated by adding 300 mol/L of H₂O₂, and changes at 470 nm were monitored. Glutathione peroxidase (GPx) activity was measured using an adaptation of the

spectrophotometric method of Flohé and Gunzler (114). This activity was determined both with H_2O_2 and cumene hydroperoxide as substrates and with glutathione reductase (GRd) and NADPH as enzyme indicators. GRd activity was measured in neutrophils by a modification of the Goldberg and Spooner spectrophotometric method which required oxidized glutathione as the substrate (127). The activities of CAT, MPO, GPx and GRd in neutrophils were determined with a Shimadzu UV-2100 spectrophotometer at 37°C. Malondialdehyde (MDA) as a marker of lipid peroxidation was analysed in neutrophils using a colorimetric assay kit (Calbiochem). Briefly, samples and standards were placed in Eppendorfs containing N-methyl-2-phenylindole (10.3 mmol/L) in acetonitrile: methanol (3:1, v/v). HCl (12 mol/L) was added and samples were incubated for 1h at 45°C. Absorbance was measured at 586 nm. The method and devices used for the oxidative parameters are presented in Table 4.

3.4.1 Materials

The method and devices used for oxidative stress parameters are presented in Table 4.

Table 4. List of methods and device used for the oxidative stress parameters.

Parameter	Sample	Method	Analyser
Superoxide dismutase	Plasma	Spectrophotometric	Shimadzu UV-2100 spectrophotometer at 37°C
Catalase	Neutrophils	Spectrophotometric	
Myeloperoxidase		Spectrophotometric	
Glutathione peroxidase		Spectrophotometric	
Glutathione reductase		Spectrophotometric	
Malondialdehyde	Neutrophils	Colorimetric assay	

3.5 Anthropometric measures and body composition assessment

Height was measured in barefoot positioning with the participant's head in the Frankfurt plane with a mobile stadiometer with 2.10 m maximum capacity and 0.001 m error margin (SECA 213, Hamburg, Germany). Weight was assessed without heavy clothing and without shoes, subtracting 0.6 g for their clothes with an electronic scale (TANITA Corp., BC-418MA, Tokyo, Japan) to the nearest 100 g. Body mass index (BMI), percentage of body fat and the muscle mass were measured by means of a portable bioimpedance analyser (Tanita Corp., BC-418MA, Tokyo, Japan) with a 200 kg maximum capacity and a ± 100 g error margin. BMI was calculated as body weight in

kilograms (kg) divided by the square of height in meters (m²). Bioimpedance analysis is inexpensive and easy to use method, and readily reproducible. Predictions equations have been validated for multi-ethnic adults (165) and reference values were established for white male and female adults, including older adults (182,183).

Waist and hip perimeter was measured in centimetres with a flexible non-elastic measuring tape (Rosscraft) to the nearest millimetre, according to the methods of the ISAK Society (210). Individuals were in a standing position with feet together and arms resting by their sides. Waist circumference (WC) was taken as the narrowest point between the inferior rib border and the iliac crest. The hip circumference (HC) measurement was taken at the point yielding the maximum circumference over the buttocks, with the tape held in a horizontal plane. Waist-to-hip ratio was calculated by dividing waist circumference (cm) by hip circumference (cm). Additionally, waist to height ratio (WHtR) was defined as waist circumference (cm) divided by height (cm). Training workshops were organized to harmonize the assessment of anthropometric measurement before starting the study in both centres. The whole harmonization process and reliability assessment has been previously published by Gómez-Cabello et al. (129).

3.5.1 Material and equipment

The materials and equipment used to collect the anthropometric and body composition data and analysis are shown in Table 5.

Table 5. Equipment used for the analyses of anthropometric and body composition.

Type of analyses	Equipment/Materials	Manufacturer/Battery
Height	Portable stadiometer SECA	Hamburg, Germany
Weight and body composition	Bioelectrical Impedance Analysis analyser TANITA BC-418	Tanita Corp., Tokyo, Japan
Waist and hip circumferences	Flexible non-elastic measuring tape (Rosscraft)	ISAK Society, South Africa (210)

3.6 Dietary assessment

Dietary intake was obtained with the 24h dietary recall by well-trained dieticians that administered the recalls. The 24h dietary recalls were collected on two non-consecutive

days within a period of 2 or 3 weeks. A photographic food album was used to facilitate the identification of the foods ingested and their portion size. The album contained photos showing foods with different sizes and presentations (130). A computer program was used to convert food into energy and nutrients (ALIMENTA®; NUCOX, Palma, Spain) based on Spanish (218,255) and European (106) food composition tables, and complemented with food composition data available for Mallorca food items (291).

3.6.1 Material and equipment

The materials and equipment used to collect the dietary intake data and analysis are shown in Table 6.

Table 6. Questionnaire and equipment used for analyses of dietary intake.

Type of analyses	Equipment/Materials	Manufacturer/Battery
24h dietary recall	2*24h dietary recall questionnaire	-Guía visual de Alimentos y Raciones (Gómez-Candela et al.) (130) -ALIMENTA®; NUCOX, Palma, Spain.

3.7 General information assessment

The general information was collected through personal interviews using a structured questionnaire that includes personal information and current and former lifestyle habits. This questionnaire has been used in the EXERNET Multi-Centre Study in Spain (269). Furthermore, quality of life was assessed using the validated Spanish version of the questionnaire EuroQol-5D-3L (29,101), which has been previously used in elderly population (252). The EuroQool-5D-3L assesses 5 different dimensions, namely mobility, self-care, daily activities, pain-discomfort and anxiety-depression. Participants were asked to indicate his/her health state in each dimension according to three different levels: (i) no problems, (ii) moderate problems, or (iii) severe problems.

3.7.1 Material and equipment

The materials and equipment used to collect the general information data and analysis are shown in Table 7.

Table 7. Questionnaire and equipment used for the analyses of general information.

Type of analyses	Equipment/Materials	Manufacturer/Battery
Personal information and current and former lifestyle habits	EXERNET questionnaire	EXERNET network http://www.spanishexernet.com/index.php

3.8 Hydration status assessment

A hydration questionnaire was developed by the Research Group based on food-frequency and eating habits questionnaires published in the literature (99,239), taking into account the modern beverage market.

The questionnaire is divided into three parts. The first part includes questions about the different type of fluids consumed one day before the questionnaire is carried out. The second part records the fluid intake during a normal week for each beverage type. The beverages included are: water, juices, soft drinks, diet drinks, milk, shake, coffee, tea or infusions, sport drinks, beer, wine and distilled drinks. The last part includes 20 questions about drinking habits. In the current thesis, the second part will be used. The description of the design has been published elsewhere (213).

3.8.1 Material and equipment

The materials and equipment used to collect the hydration status data and analysis are shown in Table 8.

Table 8. Questionnaire and equipment used for the analyses of hydration status.

Type of analyses	Equipment/Materials	Manufacturer/Battery
Hydration habits, water and beverage consumed the day before and weekly.	Hydration questionnaire® Maroto-Sánchez et al. (213)	Imfine Research Group

3.9 Physical activity and sedentarism assessment

PA was assessed by means of two validated self-reported questionnaires; the MLTPAQ measuring the activity during the last year, and the EXERNET questionnaire (ExQ), both of them validated for the Spanish population (96,97,202).

The MLTPAQ includes diverse physical activities and participants are asked about PA duration, weekly and monthly frequencies. Time for each activity was expressed in minutes per day (min/day).

The ExQ includes three types of activities: walking, sitting and household activities. Each of them could be registered under a value of daily practice time (hours): (i) < 1 h, (ii) 1-2 h (iii) 2-3 h (iv) 3-4 h (v) 4-5 h (vi) >5 h. Participants were asked to answer according to these six options pertaining to the three activities aforementioned, indicating an average time in hours for each one keeping in mind both working and weekend days. Additionally, the ExQ contains questions about regular PA. Time for each activity was expressed in minutes per day (min/day).

3.9.1 Material and equipment

The materials and equipment used to collect the PA and sedentarism data and analysis are shown in Table 9.

Table 9. Questionnaire and equipment used for the analyses of physical activity and sedentarism data.

Type of analyses	Equipment/Materials	Manufacturer/Battery
Physical activity performed during last year	Minnesota Leisure Time Physical Activity Questionnaire	Elosua et al. 1994; Elosua et al. 2000 (96,97)
Daily physical activity, sedentarism behaviour and housework	EXERNET Questionnaire	López-Rodríguez et al. (202)

3.10 Physical fitness assessment

An extended and detailed manual of operations was designed for and thoroughly read by every researcher involved in the field work before the data collection started. In order to standardize and harmonize the assessment of the PF tests, a workshop training was carried out in Madrid and Palma de Mallorca (Spain) in March 2013. The field workers were strongly advised to always perform the same fitness test in order to minimize the potential inter-rate variability within each centre. Furthermore, the instructions given to the participants in every test were standardized to ensure that the

same verbal information was given to all participants. The data logging is shown in Appendix 4.

The health-related PF components, that is, muscular strength, agility and dynamic balance and aerobic capacity were assessed by the PF tests described below. The scientific rationale for the selection of all of these tests has been published earlier and has been validated in elderly population (290). Moreover, reference values have been published for the Spanish elderly population (269).

Prior to assess PF tests, blood pressure was measured using a digital monitor. The following PF components were assessed: lower body strength by the chair stand test (290), agility/dynamic balance by the 8-foot up-and-go test (290), aerobic endurance by the 6-min walk test (290) and handgrip strength with a handgrip dynamometer (361) (Takei TKK 5401, Tokyo, Japan, range 5-100 kg, precision 0.1 kg). The handgrip strength was assessed for both hands in a standing position. All tests were performed twice, except the 6-min walk test and the chair stand test, and the best score was retained.

3.10.1 Material and equipment

The materials and equipment used to collect the PF data and analysis are shown in Table 10.

Table 10. Equipment used for the analyses of physical fitness.

Type of analyses	Equipment/Materials	Manufacturer/Battery
Lower body strength	Standardized chair (Height=43.18 cm) and stopwatch	Senior Fitness Test by Rikli & Jones
Handgrip strength	Dynamometer	Takei TKK 5401, Tokyo, Japan, range 5-100 kg, precision 0.1 kg EUROFIT battery
Agility/dynamic balance	Standardized chair (Height=43.18 cm), a cone, measuring tape and stopwatch	Senior Fitness Test by Rikli & Jones
Aerobic capacity	Seven cones, measuring tape and a stopwatch	Senior Fitness Test by Rikli & Jones
Blood pressure	Digital Automatic Blood Pressure	Omron Healthcare Co., Ltd. Kyoto, Japan

The entire description of each PF test is shown in Table 11. A final report was sent to the participants with the results obtained (Appendix 5).

Table 11. Description of the physical fitness tests performed in the PHYSMED study.

Test item	Assessment category	Description
<p>Chair stand</p>	Lower body strength	The 30-s Chair Stand Test consists of the number of full stands in 30 seconds with arms folded across chest. Initially, participants were seated on the chair with their back in an upright position. They were instructed to look straight forward and to rise after the “1, 2, 3, go” command at their own preferred speed with their arms folded across their chest (290).
<p>8-foot up-and-go</p>	Agility/Dynamic balance	Number of seconds required to rise from stated position, walk 2.45 meters, turn, and return to seated position on chair. Further instructions were to complete the test as quickly as possible (290).
<p>6-minute walk</p>	Aerobic capacity	Number of meters that can be walked in 6 minutes around a 46 meters course. Participants were instructed to walk as far as possible for 6 min (290).
<p>Handgrip strength</p>	Maximum hand strength	The participants squeeze gradually and continuously for at least 2 seconds, performing the test two times for each hand in standing position. The handgrip span was adjusted according to the hand size. The maximum score in kilograms for each hand was recorded (361).

3.11 Statistical Analysis

All data analysis was performed using the Statistical Package for Social Sciences (SPSS) version 21.0 for Windows (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp). Descriptive statistics are shown as mean $\pm$ standard deviation (SD) unless otherwise stated. P-values <0.05 were considered as statistically significant. The detailed description of statistical analysis is presented in each article.

4 RESULTS AND DISCUSSION

The results and discussion are presented below in the form in which they have been published or submitted to scientific journals, and they are referred as articles in this thesis.

4.1 ARTICLE 1: A REVIEW OF THE CUT-OFF POINTS FOR THE DIAGNOSIS OF VITAMIN B₁₂ DEFICIENCY IN THE GENERAL POPULATION

DE GRUYTER

Clin Chem Lab Med 2015; 53(8): 1149–1159

Review

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A review of the cut-off points for the diagnosis of vitamin B₁₂ deficiency in the general population

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Keywords: cut-offs; folate; homocysteine; methylmalonic acid; vitamin B₁₂ deficiency.

4.1.1 Abstract

Vitamin B₁₂ deficit is one of the most common vitamin deficiencies. However, there is no consensus on the cut-off points for vitamin B₁₂ and its co-markers, such as folate, holotranscobalamin, methylmalonic acid and homocysteine. **Methods:** In order to establish the state of the art about cut-off points used to determine vitamin B₁₂ deficiency in the last decades, the database MEDLINE was used for searching studies published in adults between December 1992 and May 2014 (69 articles), using search terms like „vitamin B₁₂“, „cobalamin“, „cut-off“, „deficiency“ alone or in combinations. **Results:** Broad ranges of cut-off points for vitamin B₁₂ and its biomarkers were identified: vitamin B₁₂ ranged between 100 pmol/L and 350 pmol/L, holotranscobalamin 20-50 pmol/L, methylmalonic acid 0.210-0.470 µmol/L, homocysteine 10-21.6 µmol/L, serum folate 3.7-15.9 nmol/L and red blood cell 124-397 nmol/L. For the majority of studies, the potential influence of age, analytical methods, sex and fortified food consumption was not taken in account when choosing cut-off values. This could explain the discrepancies between studies on vitamin B₁₂ and folate deficiency prevalences. **Conclusions:** We conclude that there is inconsistency in the literature regarding vitamin B₁₂ cut-offs. It would be necessary to establish different reference cut-offs according to age and sex, considering the analytical methods used.

Key words: cut-offs; folate; homocysteine; methylmalonic acid; vitamin B₁₂, deficiency.

4.1.2 Introduction

Vitamin B₁₂ deficiency is still an important nutritional problem worldwide as subclinical deficiency affects well defined risk groups. Vitamin B₁₂ levels decreases with age (136), which means that deficiency risk increases in parallel with age (105,200,373). Therefore, subclinical deficiency is quite more prevalent in the elderly. Epidemiologic data shows that prevalence ranges from 6% to 40% (259). However, at younger ages, a higher risk of developing vitamin B₁₂ deficiency has been described among vegetarians (150,259), patients with gastrointestinal disease (33), people with depression (91), people with high alcohol consumption (140), and people suffering from renal insufficiency (33). The main manifestations of vitamin B₁₂ deficiency are hematologic, neurologic and psychiatric disorders. Notably, vertigo, tiredness, malaise and cognitive impairment have been traditionally ascribed as „normal aging“ signs (73). Most of these disorders and symptoms are usually seen as „normal aging“ signs. However, severe vitamin B₁₂ deficiency causes an irreversible degeneration of the nervous system (132). Thus, an early diagnosis and treatment of subclinical vitamin B₁₂ deficiency is essential. In order to reach a more efficient diagnosis, a combination of several markers associated with vitamin B₁₂ metabolism could be used in place of a single vitamin B₁₂ measurement (259). The most reliable markers are serum folate (sFolate), red blood cell folate (RBC folate), holotranscobalamin (HoloTC), methylmalonic acid (MMA) and total homocysteine (tHcy).

One of the major problems when attempting to diagnose vitamin B₁₂ deficiency is the choice of the cut-off values for each of the markers. For instance, due to the high variability of cut-off values across laboratories, vitamin B₁₂ deficiency prevalence ranges from 3% for people aged ≥ 3 yr to 67.6% for elderly (71-74 yr) (274,357). This prevalence can increase up to 15.8% for institutionalized elderly, which presents a greater risk for vitamin deficiencies than the free-living elderly (136). Epidemiologic data by Andres et al. confirmed that 20% of the elderly present megaloblastic anaemia in developed countries (16). Finally, the different analytical methods used to measure vitamin B₁₂ and its co-markers introduce an additional dispersion factor for cut-off setting.

The objective of this study is to provide a review of the published biomarkers and cut-off values for the diagnosis of vitamin B₁₂ deficiency with respect to age, sex and methods of detection. The lack of consensus worldwide calls for a re-examination of the

reference values currently employed to detect mild, moderate and severe forms of vitamin B₁₂ deficiency.

4.1.3 Material and methods

The electronic database MEDLINE (<http://www.ncbi.nlm.nih.gov>) was searched for studies published between December 1992 and May 2014. Terms „vitamin B₁₂“, „cobalamin“, „cut-off“, „deficiency“ as well as combinations out of these terms were entered in the database. In addition, references in relevant articles were also used to get further information.

Only articles containing vitamin B₁₂ cut-offs were chosen. The rest of cut-offs (MMA, tHcy, HoloTC, sFolate and RBC folate) were collected when they were available. Additional data including analytical methods, geographic area, sample size and age were included if available, whereas health status of the population under study was not considered. Data on creatinine was also taken into account when authors gave this information to assess renal function. Articles were excluded if the cut-off for vitamin B₁₂ was not included, or in case of a review, a systematic review or meta-analysis.

4.1.4 Results

A total of 69 articles were included in this review. Tables 12-13 summarize the main data in each study, ordered from lowest to highest cut-off points of plasma vitamin B₁₂ concentration.

Nine studies involved a sample size ≤ 100 person, 39 studies between 100 and 500, eight articles between 500 and 1000 and 13 with more than 1000 participants. The overall number of participants was 48,868. Forty-two studies specified the sex of the participants, 18 studies did not provide sex information and nine were only performed in one sex. In terms of age, 41 studies recruited participants ≥ 60 yr and 25 studies participants ≤ 60 yr. Three studies divided the population in different age groups (173,274,357) and only one depending on racial ethnic group (274). Forty studies were carried out in European countries, 20 in American countries, four in Asiatic countries, three in oceanic countries and two in Africa.

Vitamin B₁₂

The current standard clinical screening test for vitamin B₁₂ deficiency is the determination of total vitamin B₁₂ concentration in plasma or serum. Some authors considered deficiency when vitamin B₁₂ concentration was below the low limit of indeterminate interval (grey zone), and others did not specify if they took the low limit of this indeterminate interval or the low limit of the reference range.

Cut-offs employed to define vitamin B₁₂ ranged from 100 pmol/L (102,103) to 350 pmol/L (383). Thirteen authors chose 150 pmol/L to establish vitamin B₁₂ deficiency and 12 reported a value of 148 pmol/L. Therefore, 37 % of authors used nearly the same value.

Lindenbaum et al. chose a serum vitamin B₁₂ of 258 pmol/L as a cut-off value based on high serum MMA concentrations in participants with vitamin B₁₂ values below this number (197). Wolters et al. chose a cut-off value of 258 pmol/L for their study population of 178 senior females (60-70 yr) (373). They admitted that it might be too high, because the MMA concentrations in participants in the second quartile (227-≤ 269 pmol/L) of serum vitamin B₁₂ were not significantly different from those in participants in the third (270-≤ 330 pmol/L) and fourth quartiles (<331 pmol/L). Rajan et al. suggested 221 pmol/L was a more appropriate cut-off (281). Others recommend to restructure the normal range and to raise the vitamin B₁₂ cut-off point for elderly to a much higher value than 220-260 pmol/L (247). However, recently, Valente et al. used the lower limit of the 95 % central reference interval (123 pmol/L) as established from their reference population (352).

Holotranscobalamin

Cut-offs for HoloTC ranged between 20 and 50 pmol/L. The lower one was established by Valente et al. (352) by using FPIA measurement and the higher one was set by Hvas and Nexø (160), Bor et al. (48) and Lloody-Wright et al. (199) using CPB and ELISA analytical methods, respectively. The principal methodology run was RIA assay (65 %) and the corresponding cut-off range was between 35 and 40 pmol/L. Only 20 authors considered HoloTC in their studies.

Methylmalonic acid

A total of 44 articles included MMA as biomarker to assess vitamin B₁₂ deficiency. Reported values ranged from 0.210 to 0.470 µmol/L. The majority of GC-MS users (77.3%) reported very close cut-offs: from 0.26 to 0.28 µmol/L. Only one author disclosed different MMA cut-off for adults and elderly (357).

Homocysteine

tHcy was measured in 53 studies and cut-off values ranged from 10 µmol/L (383) with HPLC to 21.6 µmol/L with GC-MS (270). Only seven studies did not specify the analytical method used. FPIA was the preferred method (39%) to determine tHcy levels, followed by HPLC (32.6%) and GC-MS (21.7%). Fifteen studies set tHcy cut-off at 15 µmol/L and this value was the most commonly used. Two studies divided samples by sex, reporting 17.1 µmol/L and 16.8 µmol/L (62), and 11.4 µmol/L and 10.4 µmol/L for males and females, respectively (120).

sFolate

This variable was measured in 40 studies and the method mostly used for analysis was RIA (53%). The most common cut-off used was 6.8 nmol/L (22.5% of studies), followed by 7 nmol/L (17.5%), although values ranged from 3.7 nmol/L (RIA) to 15.9 nmol/L (MEIA).

RBC folate

Only 14 articles (20.6%) included RBC folate measurement. All studies used different cut-off concentrations which ranged between 124 nmol/L (CLIA) and 397 nmol/L (MEIA). The most common methodology was CLIA.

Creatinine

Creatinine values were separated by sex in 13 articles, reporting a cut-off range from 90 µmol/L to 141 µmol/L for males and 79.6 µmol/L to 120 µmol/L for females. Fifteen articles did not separate data by sex, giving a cut-off interval from 97 µmol/L to 141 µmol/L. The majority of studies have considered 120 µmol/L or 124 µmol/L as high reference range limit for males and 115 µmol/L for females.

Table 12. Overview of different characteristics used in selected studies, sorted by the concentration of vitamin B₁₂ cut-off.

No.	References	City (country) ^a	n (all; m, f)	Age (yr)
1	(102)	Wageningen (NL)	120	≥70
2	(103)	Germany (DE)	202; 52, 150	81±6 ^h
3	(173)	(BE, DE, NL)	1=99; 53, 46 2=64; 20, 44 3= 286; 115, 171	1=young subpopulation 19-55 2= healthy elderly people 65-88 3= hospitalized elderly 61-97
4	(243)	13 centres (BE)	285; 105, 180	65-96 ^d
5	(40)	Skutskär (SE)	224; 94, 130	78 (77.2-78.9) ^c
6	(284)	Toronto (CA)	711; 403, 308	58 ^f
7	(352)	Dublin (IE)	700; 210, 490	63-97
8	(139)	Cardiff (GB)	49	< 75
9	(115)	Sydney (AU)	2963	≥ 50
10	(48)	Copenhagen (DK)	98; 0, 98	41-75 ^d
11	(150)	Saarland (DE)	232; 79, 153	69 (19-102) ^g
12	(199)	Oxford (GB) London (GB)	195; 195, 0	44 (18-78) ^g
13	(320)	Finland (FI)	2806; 1328, 1478	45-74 ^d
14	(62)	Los Angeles (US)	591; 345, 246	>60
15	(113)	Perth (AU)	299	≥75
16	(109)	Tel-Hashomer (IL)	1167	≥69
17	(16)	Strasbourg (FR)	201; 57, 144	67±6 ^h
18	(359)	Florida (US)	359; 0, 359	20-30 ^d
19	(194)	Gothenburg (SE)	209	76 (70-93) ⁱ
20	(231)	Sacramento (US)	1789; 751, 1038	≥60
21	(237)	United States (US)	1145	≥65
22	(136)	Granada (ES)	218; 82, 136	79.2 (60-105) ^g
23	(259)	Granada (ES)	218; 82, 136	65-90 ^d
24	(236)	Boston (US)	1458	70±0.32 ^h
25	(311)	Santiago de Chile (CL)	491	65-67.9 ^d
26	(332)	New Heaven (US)	255	28-82 ^d
27	(267)	Ontario (CA)	75; 28, 47	80.7±1.5 ^h
28	(197)	New York (US)	548; 200, 348	77.5 (67-96) ⁱ
29	(274)	Unites States (US)	7233; 3689, 3544	≥3
30	(107)	Adelaide (AU)	64; 64, 0	50-70 ^d
31	(201)	Lieto (FI)	224; 92, 132	≥65
32	(105)	Lausanne (CH)	50; 0, 50	18-91 ^d
33	(247)	Lund (SE)	209; 91, 118	73±11 ^h
34	(287)	Puna (IN)	204; 169, 35	27-55 ^d
35	(289)	Boston (US)	70; 70, 0	54-81 ^d
36	(329)	Amsterdam (NL)	1; 1, 0	51
37	(374)	Busan (KR)	184; 94, 90	61. 6 (22-83) ⁱ
38	(83)	Leiden (NL)	423	≥85
39	(73)	Oxford (GB)	1562; 618, 884	≥65
40	(72)	Great Britain (GB)	1549	≥65
41	(200)	Lieto (FI)	1048	65-100 ^d
42	(354)	Nijmegen (NL)	105; 46, 59	76 (74- 80) ⁱ
43	(120)	Araihasar (BD)	1650; 677, 973	20-65 ^d
44	(187)	Ibadan (NG)	139; 61, 78	60-98 ^d
45	(175)	India (IN)	36	50.6 (16-80) ⁱ
46	(149)	Saarland (DE)	545; 251, 294	57 (18-92) ^g

No.	References	City (country) ^a	n (all; m, f)	Age (yr)
47	(151)	Santiago (CL)	108; 41, 67	74.4±3.7 ^h
48	(154)	Oslo (NO)	224	18-90 ^d
49	(334)	Uganda (UG)	280	>18
50	(339)	Leiden (NL) Glasgow (GB)	185; 88, 97	≥65
51	(251)	Homburg (DE)	228; 72, 156	>65
52	(159)	Aarhus (DK)	937; 349, 588	72 (19-102) ⁱ
53	(160)	Aarhus (DK)	143; 51, 92	72 (24-90) ⁱ
54	(198)	Gothenburg (SE)	101; 35, 66	52 (1-80) ⁱ
55	(246)	Haukeland (NO)	90; 69, 21	38- 80 ^d
56	(286)	Oxford (GB)	116; 43, 73	73 ⁱ
57	(74)	Oxford (GB)	2403;986, 1417	≥65
58	(357)	Norway (NO)	6946; 3075, 3871	(47-49 adults) ^d (71-74 elderly) ^d
59	(180)	Albuquerque (US)	100; 50, 50	68- 96 ^d
60	(270)	Denver (US)	152	65-99 ^d
61	(281)	Seattle (US)	315; 203, 112	65-100 ^d
62	(206)	Mashhad (IR)	244; 126, 154	≥65
63	(172)	Georgia (US)	103; 21, 82	76±8 ^h (60-95) ^d
64	(68)	(US) and (ES)	1350; 656, 694	65-90
65	(292)	London (GB)	421; 215, 206	66 (37-90) ^g
66	(335)	Baltimore (US)	762; 0, 762	≥65
67	(179)	Hanover (DE)	178; 0, 178	60-70 ^d
68	(344)	Chicago (US)	121	≥65
69	(383)	Molise (IT)	240; 161, 79	18-66

^a, ISO 3166-1 country code elements; ^b, Abbreviations: m, male; f, female. To convert vitamin B₁₂ (pg/ml) to pmol/L, multiply with 0.738; vitamin B₁₂ and HoloTC to ng/L, multiply with 1.3554; folate to ng/mL, multiply with 2.266; MMA to mg/L, divide by 8.475; tHcy to mg/L, divide by 7.397; creatinine to g/dL, divide by 88.4; ^d, range; ^e, mean (95 % CI); ^f, mean; ^g, mean (range); h. mean ± SD; ⁱ, median (range).

Table 13. Overview of different cut-offs used to define vitamin B₁₂ deficiency in selected studies, sorted by the concentration of vitamin B₁₂ cut-off.

No.	References	B ₁₂ ^{bc} (pmol/L)	HoloTC ^{bc} (pmol/L)	MMA ^{bc} (μmol/L)	tHcy ^{bc} (μmol/L)	sFolate ^{bc} (nmol/L)	RBC folate ^{bc} (nmol/L)	Creati- nine ^c (μmol/L)
1	(102)	100 CLIA		0.260 GC-MS				120
2	(103)	100 ND		0.320 ND				120
3	(173)	103 RIA		0.247 GC-MS	13.9 GC-MS	5.4 RIA		124
4	(243)	103 RIA		0.247 GC-MS	13.9 GC-MS	5.4 RIA		
5	(40)	115 RIA		0.370 ND	15 ND	3.7 RIA	275 ILA	
6	(284)	120 CPB			15 HPLC		215 CPB	120
7	(352)	123 MBA	20 FPIA	0.36 GC-MS	15 FPIA	6.8 MBA	340 MBA	
8	(139)	125 EIA	38 RIA	0.470 GC-MS	15 FPIA			
9	(115)	125 ND			15 ND	6.8 ND		
10	(48)	130 CLIA	50 ELISA	0.280 GC-MS	11.9 FPIA			115 (f)
11	(150)	132 ND		0.271 ND	15 ND	6.4 ND		106
12	(199)	135 EIA	50 ELISA	0.280 GC-MS	12 GC-MS	6.8 EIA		
13	(320)	138 CLIA						
14	(62)	140 RA		0.376 HPLC	17.1 (m) 16.8 (f) HPLC	5.7 RIA		133 (m) 115 (f)
15	(113)	140 ND			15 ND			
16	(109)	147 ELISA		0.240 HPLC	15 RIA	11 ELISA		141
17	(16)	148 RIA			13 GC-MS	6.8 ND		120
18	(359)	148 RIA	35 RIA		14 HPLC			
19	(194)	148 RIA		0.340 GC-MS	16 HPLC	8.6 RIA		
20	(231)	148 RIA	35 RIA	0.350 LC-MS	13 HPLC		363 CLIA	124 (m) 97 (f)
21	(237)	148 RIA		0.370 GC-MS				133(m) 115 (f)
22	(136)	148 MEIA	45 RIA	0.300 GC-MS	12 FPIA	6 MEIA	397 MEIA	
23	(259)	148 MEIA	35 RIA	0.300 GC-MS	13 FPIA	15.9 MEIA	≤ 396.4 MEIA	

No.	References	B ₁₂ ^{bc} (pmol/L)	HoloTC _b (pmol/L)	MMA ^{bc} (μmol/L)	tHcy ^{bc} (μmol/L)	sFolate ^{bc} (nmol/L)	RBC folate ^{bc} (nmol/L)	Creati- nine ^c (μmol/L)
24	(236)	148 RIA		0.210 GC-MS				131(M) 115 (F)
25	(311)	148 RIA				7 RIA		
26	(332)	148 CLIA		0.376 GC-MS	12.2 FPIA			
27	(267)	148 CLIA			13.3 FPIA		370 CLIA	
28	(197)	148 RIA		0.376 GC-MS	21.3 GC-MS	5.9 RIA		124 (m) 106 (f)
29	(274)	148 RIA		0.370 GC-MS	9 FPIA	6.8 RIA		
30	(107)	150 RIA			10 HPLC	6.8 RIA	317 RIA	
31	(201)	150 CPB	37 RIA	0.450 GC-MS	19.0 FPIA			
32	(105)	150 RIA		0.260 GC-MS		7 RIA		97
33	(247)	150 TR-FIA	40 RIA	0.410 GC-MS	19.9 HPLC	7 RIA		120
34	(287)	150 RIA	35 RIA	0.260 GC-MS	15 HPLC	5 RIA		110
35	(289)	150 RIA			18.5 HPLC	6.8 RIA		
36	(329)	150 MEIA			18 FPIA	6.5 MEIA		
37	(374)	150 MEIA	35 ND		12 FPIA	6.8 ND		
38	(83)	150 DCSP			13.5 FPIA	7 DCSP		
39	(73)	150 CPB		0.350 GC-MS	15 FPIA	5 MBA		100
40	(72)	150 CPB			20 FPIA	7 MBA		
41	(200)	150 CPB	37 RIA		≥15 FPIA			
42	(354)	150 RIA		0.320 GC-MS	19.9 HPLC			90 (m) 110 (f)
43	(120)	151 RIA			11.4 (m) 10.4 (f) HPLC	9 RIA		
44	(187)	151 HPBC						
45	(175)	155.7 CLIA						
46	(149)	156 CLIA	35 RIA	0.271 GC-MS	12 GC-MS	7 CLIA		
47	(151)	165 IAC			14 FPIA	6.8 IAC		
48	(154)	170 RIA		0.376 CE	15.0 HPLC	5 RIA		125 (m) 115 (f)
49	(334)	177 ND						
50	(339)	184 CLIA					124 CLIA	

No.	References	B ₁₂ ^{bc} (pmol/L)	HoloTC _b (pmol/L)	MMA ^{bc} (µmol/L)	tHcy ^{bc} (µmol/L)	sFolate ^{bc} (nmol/L)	RBC folate ^{bc} (nmol/L)	Creati- nine ^c (µmol/L)
51	(251)	196 CLIA	29 RIA	0.280 GC-MS	14.1 GC-MS	11.1 CLIA		106.1 (m) 79.6 (f)
52	(159)	200 CPB	40 RIA	0.280 GC-MS	11.9 FPIA		350 CPB	133 (m) 120 (f)
53	(160)	200 CPB	50 CPB	0.290 GC-MS	5 FPIA		50 CPB	133 (m) 115 (f)
54	(198)	200 CPB	35 ND	0.400 GC-MS	13 HPLC			
55	(246)	200 EIA	40 ELISA	0.280 ND				
56	(286)	200 ND	40 RIA	0.300 GC-MS	14 ND	15 ND		115
57	(74)	200 CLIA		0.45 GC-MS				124 (m) 97 (f)
58	(357)	150;200; 400 MBA		0.280 adults 0.360 elderly GC-MS				106 (m) 87 (f)
59	(180)	221 ND		0.271 ND	16.2 ND	5 ND		
60	(270)	221 RA		0.376 GC-MS	21.6 GC-MS			
61	(281)	221 ND		0.271 ND	13.9 ND	7.3 ND		109
62	(206)	243.5 RIA			15 ELISA	14.7 RIA		
63	(172)	258 RIA		0.271 GC-MS	13.9 GC-MS	6.8 RIA	295 RIA	127
64	(68)	258 ND			15 CLIA	14 ND		
65	(292)	258 EIA		0.271 GC-MS	14 HPLC			
66	(335)	258 CPB		0.271 GC-MS	13.9 GC-MS	11.4 CPB		100
67	(179)	258 CLIA		0.271 GC-MS	12 FPIA	7 CLIA	320 CLIA	
68	(344)	258 CDI		0.271 GC-MS				
69	(383)	350 ILA			10 HPLC	15 ILA	305 ILA	

^a, ISO 3166-1 country code elements; ^b, Abbreviations: m, male; f, female; CE, capillary electrophoresis; CLIA, chemiluminescent immunometric assay; CDI, competitive displacement immunoassay; CPB, competitive protein binding; DCSP, dual count solid phase no boil-assay; EIA, enzyme Immunoassays; ELISA, Enzyme-Linked ImmunoSorbent assay; FPIA, fluorescence polarization immunoassay; GC-MS, gas chromatography- mass spectroscopy; HPLC, high pressure liquid chromatography; IAC, ion capture assay; ILA, immunoligand assay; LC-MS, liquid chromatography- mass spectroscopy; MBA, microbiological assay; MEIA, microparticle enzyme immunoassay; RA, radiodilution assay; RIA, radioimmunoassay; TRFIA, time-resolved fluoroimmunoassay; ND, there was no date or no specific method; ^c, SI conversion factors: To convert vitamin B₁₂ (pg/ml) to pmol/L, multiply with 0.738; vitamin B₁₂ and HoloTC to ng/L, multiply with 1.3554; folate to ng/mL, multiply with 2.266; MMA to mg/L, divide by 8.475; tHcy to mg/L, divide by 7.397; creatinine to g/dL, divide by 88.4; ^d, range; ^e, mean (95 % CI); ^f, mean; ^g, mean (range); h. mean ± SD; ⁱ, median (range).

4.1.1 Discussion

A major problem when comparing vitamin B₁₂ deficiency prevalence across studies consisted on the variability of cut-off values used. Several factors can be addressed in this context. The first one was the method used to establish cut-offs values since means, medians, two or three SD or percentiles and the analytical methods used to measure biomarkers were diverse. Another issue was the application of values obtained from younger participants to the elderly. All these factors contributed to scattered cut-off values. This is the first review to group information on cut-off values for vitamin B₁₂ deficiency from 1992 to 2014, taking into account age and analytical detection methods. Regarding the literature, the prevalence of vitamin B₁₂ deficiency in British elderly ranged from 5% (age 65-74 yr) to 10% (age >75 yr) with 150 pmol/L as a cut-off (72). Sanchez et al. found 12% B₁₂ deficiency using 148 pmol/L and 25.4% with cut-off value of <221 pmol/L (311). Meanwhile, Palacios et al. (259) found 17.4% ($\leq$ 148 pmol/L) and Loikas et al. (200) observed 6.1% considering 150 pmol/L as cut-off. Two extreme B₁₂ cut-offs were recorded: the lowest one (123 pmol/L) was used by Valente et al. and observed 8% of vitamin B₁₂ deficiency (352). However, Zappacosta et al. using the highest value (350 pmol/L) found that only 16.3% of the Italian adults presented an adequate vitamin B₁₂ concentration (383). A detailed study on vitamin B₁₂ status was carried out by Vogiatzoglou et al. using 150 pmol/L as cut-off; they found a prevalence of 0.4% for adults and 1.7% for elderly (357).

Only four authors used vitamin B₁₂ as a single marker, while the rest of them made a combination of markers, as already proposed by Valente et al. (352) and Palacios et al. (259). The biomarker most included was tHcy (78%) followed by MMA (65%), sFolate (59%), HoloTC (30%) and RBC folate (21%). The small number of studies including RBC folate was unexpected, as erythrocyte folate is considered a more precise physiological marker of folate concentrations than sFolate. This may be due to the preparation of samples for RBC folate measurement which requires more laborious steps than for sFolate. Additionally, both folate markers should be measured to assess if there is a mixed deficiency between folate and vitamin B₁₂ (136). However, MMA measurement is important to distinguish between vitamin B₁₂ and folate deficiency. Elevated MMA is a specific marker of vitamin B₁₂ deficiency while tHcy rises in both vitamin B₁₂ and folate deficiencies (52,373). The majority of studies did not take into account age or sex of participants when they applied the cut-off values. For example,

none of the studies modified the cut-offs for vitamin B₁₂ depending on age and only two for MMA. Vogiatzoglou et al. set different cut-off values for middle-ages (47-49 yr; 0.280 µmol/L) and elderly (71-74 yr; 0.360 µmol/L) due to different creatinine and vitamin B₁₂ concentrations (357). In the same way, Clarke et al. took in consideration both age and renal function to adapt cut-offs because their study was carried out in elderly (73). In the elderly, impaired renal function could be an important confounding factor for tHcy and MMA (80). For this reason, we have included in the Tables 12-13 the creatinine cut-off values when available. In the same way, none of the studies included different cut-offs for tHcy depending on age. Only Carmel et al. (62) and Gamble et al. (120) reported values for males (m) and females (f) separately: 17.1 µmol/L (m) and 16.8 µmol/L (f), and 11.4 µmol/L (m) and 0.4 µmol/L (f). Age and sex did not seem to influence the choice of cut-offs values for both sFolate and RBC folate. MMA was predominantly measured using GC-MS methods. This method is expensive and not available in all the routine laboratories. Therefore, Palacios et al. proposed an algorithm to differentiate vitamin B₁₂ from folate deficiency with no need for MMA measurement (259).

Countries that currently mandate folic acid fortification as well as individuals under multivitamin supplementation may require specific reference values for the diagnosis of vitamin B₁₂ deficiency (Folic Acid Mandatory Fortification). Vitamin B₁₂ supplements or food fortification should be taken into account in countries that use this kind of supplementation. A study by Sanchez et al. showed that a daily dose of 1.4 µg of vitamin B₁₂ was not sufficient to improve vitamin B₁₂ status (311). Supplementation with higher doses of vitamin B₁₂ (>500 µg/day) have proven effective in Latino populations residing in USA (59) and institutionalized Spanish elderly increased significantly from 308.4 pmol/L to 558.3 pmol/L ($p < 0.001$) (6). Also, Favrat et al. showed that oral vitamin B₁₂ treatment normalized the metabolic markers (105). However, this response did not persist for an additional 3 months following cessation of therapy. These discrepancies may be the result of employing the same cut-offs for diagnosing vitamin B₁₂ deficiency in populations exposed to different basal levels of vitamin B₁₂ and folate. Vitamin B₁₂ is not the only vitamin affected since folate is also added to food in some countries. In these countries with food folate fortification or populations taking folic acid supplements, the upper reference limit for tHcy is usually 20%-25% lower than in non-fortified populations (286). Clarke et al. suggested as

ultimate gold standard for vitamin B₁₂ deficiency the reduction in increased tHcy or MMA concentrations and improvement in clinical symptoms or signs in response to vitamin B₁₂ treatment (73).

Limitations of this review comprise the lack of information on preanalytical procedures, analytical performance and diagnostic accuracy, even if these factors may have a deep impact on cut-off values choice. Unfortunately, the majority of articles did not specify these kinds of data. Therefore, we were not able to include them in our review.

4.1.2 Conclusions

There is no consensus in the literature about cut-off points for blood vitamin B₁₂ reference ranges and its associated metabolites. In most of the studies, age and sex were not taken into account when measuring any of these biomarkers, nor the use or consumption of fortified food. Published data in the literature regarding deficiency percentage should be considered with caution. For future studies on vitamin B₁₂ status, it would be necessary to establish different reference cut-offs according to age and sex, considering the analytical methods used.

4.2 ARTICLE 2: A NOVEL PHYSICAL ACTIVITY AND SEDENTARY BEHAVIOR CLASSIFICATION AND ITS RELATIONSHIP WITH PHYSICAL FITNESS IN SPANISH OLDER ADULTS. THE PHYSMED STUDY.

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A Novel Physical Activity And Sedentary Behavior Classification And Its Relationship With Physical Fitness In Spanish Older Adults. The PHYSMED Study.

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4.2.1 Abstract

As there is not a gold standard methodology to classify older people in relation to physical activity (PA) and sedentary behaviour, this paper aimed to propose a classification combining PA and sedentary behaviour. **Methods:** Within a broader study, 433 participants, aged over 55 yr (57% females) from Madrid and Mallorca, were evaluated for PA and sedentary behaviour by means of validated questionnaires. Physical fitness was analysed objectively using the EXERNET test battery. Cluster analyses were used to establish behavioural patterns, combining PA and sedentary behaviour. **Results:** Males spent more time doing regular PA but less time walking and working at home than females ($p<0.001$). Comparing the groups: inactive and high sedentary, inactive and low sedentary, active and high sedentary and active and low sedentary, the worst aerobic endurance ($p<0.001$), and lower body strength ($p<0.05$) were obtained in males from both inactive groups. Agility was highest in the active and low sedentary group ($p<0.05$). No significant differences were observed in females. **Conclusions:** The proposed classification is valid as it classifies participants according to their PA and sedentary behaviour and outcomes are related to objectively measured fitness. It could facilitate the work of public health authorities, researchers and physicians.

Key words: physical fitness; physical activity; sedentary lifestyle; aging.

4.2.2 Introduction

Currently, it is well established that active people show lower rates of all-cause mortality and lower risk of suffering from coronary heart disease, high blood pressure, stroke, type 2 diabetes, metabolic syndrome, colon cancer, breast cancer and depression (70,123,375). But this said, there is an ongoing discussion in the literature to clarify a) if it is physical activity (PA) or physical fitness (PF) which is the main influence in health-related factors (43,302), b) which of them should be measured or addressed in scientific studies and clinical care (44), and c) which of the terms should be used (42). Although both terms are closely related, they should not be treated as synonyms (42). PA is defined as any body movement performed by muscle action that increases energy expenditure (256). PA can be quantified by intensity, duration or frequency, using participative or objective measurements. Moreover, PA volume is calculated by different units such as metabolic equivalents (METs), minutes of weekly PA, kilocalories expended per kilogram of weight per day, minutes of participation and frequency of PA during the week (340).

On the other hand, PF is the capacity to perform PA and integrates the body functions (cardio-respiratory, skeleto-muscular, hemato-circulatory and endocrine-metabolic), and also a wide range of physiological and psychological qualities (256). Additionally, genetic contributions to fitness are important (340). PF is usually measured by means of validated objective tests (203,269,290), and can be expressed by each of the capacities individually or by summing them up in a score (271). According to several studies, PF is a stronger predictor of cardiovascular and all-cause mortality than other risk factors (even PA) and it plays an important role as a determinant health marker (256,269,290). Specifically in elderly, a high PF level has been proposed to extend years of active independent living, reducing disability and improving quality of life (340). Therefore, PF is proposed as the gold standard method, and the best marker of long term health (43,302).

Independently of the debate over PA or PF, prolonged sitting has emerged as a risk factor for early mortality and seems to have deleterious health effects (39,219). Sedentary behaviours include sitting during commuting, at the workplace, in the domestic environment, and during leisure time. A total of 41.3% of the Spanish population (337), 42% of Europeans and globally around 31% of adults aged above 15 yr claim to be sedentary or never perform exercise or play sport (381). Furthermore, low

active, sedentary and inactive have been used as synonyms in the literature, but these words seem not to be the same (381). There is the possibility that individuals can be both sedentary and physically inactive; there is also the potential for high sedentary time and being physically active (257). Most of the studies have evaluated PA and sedentary behaviour separately, but there are data in the literature indicating that both aspects should be approached in a combined way (257). These different terminologies and kinds of measurements do not facilitate a standardized classification. Specifically in older adults, several have shown that the amount of PA performed is not the most accurate method for defining the physical condition of older people and accordingly, their ability to carry out daily activities in an independent manner (65,98,290,312). Regarding sedentary behaviour, to the best of our knowledge, data are scarce on elderly. Hence, a key point to define public health recommendations is to be able to classify people according to their PA, PF or even sedentary behaviour. The proportion of the elderly population is increasing globally and it has become a priority for public health authorities (171).

Therefore, the aim of this study was to propose a classification for older people combining PA and sedentary behaviour as behavioural patterns and to study the association of these with PF.

4.2.3 Material and Methods

Study design, sample and ethics

The present study was based on a cross-sectional multi-centre study aimed at identifying cardiovascular risk factors in sedentary and active older participants. Data collection took place from April 2013 to May 2014 in Madrid and Mallorca (Spain). The recruitment of participants for the study was carried out by snowball system through health centres, sport federations, sport facilities and municipal clubs. The present study sample consisted of 477, however, 25 participants declined the participation and another 19 did not complete all the tests. A total of 433 participants, 186 male (43%) and 247 females (57%) aged 55 to 88 yr were included in this study. The exclusion criteria were age under 55 yr, being institutionalized, suffering from a physical or mental illness which would have limited their participation in the PF tests or their ability to respond to the questionnaires or drug intake for clinical research. The study was performed according to the principles established in the Declaration of

Helsinki and approved by the Ethical Committee of the Technical University of Madrid. Written informed consent was obtained from each participant.

Physical activity and sedentary behaviour self-report

PA was assessed by means of two self-reported complementary questionnaires; the Minnesota Leisure Time Physical Activity Questionnaire (MLTPAQ) measuring activity during the last year, and the EXERNET questionnaire (ExQ), asking about specific daily hours of activity, both of them validated for the Spanish population (96,97,202). The questionnaire data were collected using trained data collectors.

MLTPAQ includes different physical activities and participants are asked about exercise duration, weekly and monthly frequencies. Time for each activity is expressed in minutes per day (min/day). Afterwards, activities were summed and grouped together and classified into two groups: time performing regular physical activity (TPA_MLTPAQ) and time spent walking combined with time doing housework (TWH_MLTPAQ).

On the other hand, the validated ExQ included three questions about time spent walking, time spent doing housework and time spent sitting (TSS) per day and these questions were bounded. Answers included six options which were classified as less than 1 h, between 1 and 2 h, between 2 and 3 h, between 3 and 4 h, between 4 and 5 h and more than 5 h. Also, another question was about regular PA and participants responded on number of hours per week. Following the same criteria described above, all activities were recorded in min/day, summed and classified into three groups: TPA_ExQ, TWH_ExQ and TSS_ExQ (time spent sitting).

Physical fitness tests

Each participant completed a multi-component battery of PF test validated in older people population (290) and validated reference ranges for Spanish older adults proposed by Pedrero-Chamizo et al. (269). Lower body strength by the chair stand test (290), agility/dynamic balance by the 8-foot up-and-go test (290), aerobic endurance by the 6-min walk test (290) and handgrip strength with a dynamometer (Takei TKK-5401, Tokyo, Japan, range 5-100 kg, precision 0.1 kg) (361). The handgrip strength was assessed for both hands in a standing position. All tests were performed twice, except the 6-min walk test and the chair stand test, and the best score was retained.

Anthropometric measurements

Height was measured to the nearest millimetre using a mobile stadiometer (SECA 213, Germany), with the participant's head in the Frankfurt plane. Weight was assessed by means of bioimpedance analysis (TANITA Corp, BC-418MA, Tokyo, Japan) in standardized conditions. Also, BMI was calculated as weight (kg)/height² (m). Anthropometric measurements were performed by a trained ISAK level II anthropometrist in order to minimize the inter-observer coefficients of variation.

Lifestyle variables

ExQ also contained questions about educational level and smoking habits. The questions were grouped as follows: (i) educational level: primary school, secondary school and university graduate; and (ii) smoking habits: smoker and non-smoker.

Statistical analysis

Statistics SPSS 21.0 software was used to analyse data (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp). All variables of PF tests were checked for normality of distribution with the Kolmogorov-Smirnov test. All PF tests showed a normal distribution. The results of each PF tests were stratified by sex and age groups (distributed in five years periods without truncating the last group). Regarding PA and sedentary data, principal components Analysis for Categorical Data was conducted to observe if there were correlations between the different sets of parameters (TPA and TWH) measured by ExQ and MLTPAQ, and the relationship between TPA and TWH with TSS. Due to the correlation obtained between TPA and TWH in both questionnaires ($r=0.63$ and $r=0.55$, respectively, $p<0.05$), the following set of parameters was used together for cluster analyses (Figure 5) in order to define behaviour patterns: (1) TPA from MLTPAQ and ExQ; (2) TWH from MLTPAQ and ExQ; (3) TSS from the ExQ. These parameters were also analysed to evaluate the influence of sex and we conducted separate cluster analyses for males and females to reflect sex-based differences in PA patterns. Because clusters were consistent across sexes, we chose not to stratify by sex in order to preserve sample size for clustering. Thus, k-means clustering was used in all clusters.

TPA and TWH clusters were divided into 3 levels: low, medium and high and these were scored from 1 (low level) to 3 (high level) points. Scores of TPA and TWH were

added together and the maximum possible score was 6 points. Then, the total score was divided in two groups: inactive (2-3 points) and active (4-6 points). On the other hand, TSS cluster was divided into low sedentary (≤ 3 h) and high sedentary (> 3 h) using analyses of clustering.

In order to classify our population taking into account analyses of clustering, we established four groups: inactive and high sedentary, inactive and low sedentary, active and high sedentary and active and low sedentary. Furthermore, one-way ANOVA was performed to study the behaviour patterns on physical fitness. *Post hoc* analyses were conducted with the Bonferroni adjustment, divided by sex and controlled by age. Level of significance for acceptance was $p < 0.05$.

MLTPAQ, Minnesota Leisure Time Physical Activity Questionnaire; ExQ, EXERNET questionnaire, TPA, time performing regular physical activity; TWH, time spent walking combined with time doing housework; TSS, time spent sitting.

Figure 5. Description of the population classification.

4.2.4 Results

Descriptive data are shown in Table 14. The mean age of the participants was 65.4 ± 6.6 yr for males and 67.5 ± 6.6 yr for females ($p < 0.01$). The mean of BMI was 27.5 kg/m^2 and 26.7 kg/m^2 for males and females, respectively. Around 50% of females were low educational level (primary school) and the majority of the participants ($>90\%$) were non-smokers. Males spent more time doing regular physical activity than females ($p < 0.001$) in both questionnaires, but less time walking and working at home in MLTPAQ ($p < 0.001$). Comparing TWH and TPA obtained with the two questionnaires, minutes spent doing TPA were higher with MLTPAQ, in both sexes ($p < 0.001$).

Table 14. Descriptive data and scores obtained from different questionnaires by sex.

	Male (n=186)	Female (n=247)	p
Age (yr) *	65.4 ± 6.6	67.5 ± 6.6	<0.01
Height (cm) *	170.2 ± 6.6	156.6 ± 5.6	<0.01
Weight (kg) *	79.9 ± 10.8	65.4 ± 10.4	<0.01
BMI (kg/m^2) *	27.5 ± 3.1	26.7 ± 4.3	<0.05
City†			
Madrid	78 (49.1)	122 (49.4)	>0.05
Mallorca	108 (58.1)	125 (50.6)	
Educational level†			
Primary school	67 (36.0)	121 (48.9)	<0.05
Secondary school	59 (31.7)	71 (28.8)	
University graduate	60 (32.3)	55 (22.3)	
Smoking habits†			
Yes	16 (8.6)	18 (7.3)	>0.05
No	170 (91.4)	229 (92.7)	
TWH_MLTPAQ (min/day) *	109.5 ± 78.6	136.0 ± 78.5	<0.001
TPA_ExQ (min/day) *	35.2 ± 38.1	24.8 ± 26.2	<0.001
TPA_MLTPAQ (min/day) *	52.0 ± 52.0	32.4 ± 35.1	<0.001
Chair stand test (rep)	15.6 ± 3.4	14.1 ± 3.3	<0.001
Agility and dynamic balance (s)	4.8 ± 0.9	5.3 ± 1.0	<0.001
Aerobic endurance (m)	650.0 ± 105.0	559.4 ± 81.0	<0.001
Handgrip strength (kg)	37.9 ± 6.9	21.8 ± 4.6	<0.001

Values are expressed as follows: *mean $\pm$ standard deviation; †n (%). TWH_MLTPAQ, time spent walking and time doing housework by Minnesota questionnaire; TPA_ExQ, time performing regular physical activity by EXERNET questionnaire; TPA_MLTPAQ, time performing regular physical activity by Minnesota questionnaire; BMI, body mass index. Significant differences between means by one way ANOVA. Level of significance $p < 0.05$.

Table 15 shows cluster characteristics split by sex. A total of 69.9% of males and 57.1% of females had high levels of sedentary behaviour. Also, 62.8% of females presented a low level of TPA; conversely, 71% of males had a low level of TWH.

Table 15. Characteristics of each cluster by sex.

		Male (n=186) ¹	Female (n=247) ¹
Cluster TPA	Low	44.6	62.8
	Medium	37.1	30.4
	High	18.3	6.9
Cluster TWH	Low	71.0	43.3
	Medium	19.4	44.1
	High	9.7	12.6
Cluster TSS	Low	30.1	42.9
	High	69.9	57.1
Inactive and high sedentary		48.9	42.1
Inactive and low sedentary		16.1	27.9
Active and high sedentary		21.0	15.0
Active and low sedentary		14.0	15.0

Cluster TPA, time performing regular physical activity; Cluster TWH, time spent walking and time doing housework; Cluster TSS, time spent sitting. ¹Values are expressed as follow: %.

Table 16 presents the correlation between TPA_ExQ and TPA_MLTPAQ, TWH_ExQ and TWH_MLTPAQ, TSS_ExQ of both questionnaires and each group with sex. There was a positive and significant correlation between TPA and TWH in both questionnaires ($r=0.63$ and $r=0.55$, respectively, $p<0.05$), but none of the TPA or TWH groups correlated with TSS. No differences were found between TPA, TWH and TSS from questionnaires regarding sex.

Table 16. Cross-correlation of EXERNET and Minnesota questionnaires for time performing regular physical activity, time spent walking and time doing housework and time spent sitting.

	TWH_ExQ	TPA_ExQ	TSS_ExQ	TWH_MLTPAQ	TPA_MLTPAQ	Sex
TWH_ExQ	1.00	-0.18	-0.22	0.55*	-0.18	0.35
TPA_ExQ	-0.18	1.00	-0.15	-0.10	0.63*	-0.13
TSS_ExQ	-0.22	-0.15	1.00	-0.19	-0.17	-0.17

*Level of significance $p<0.05$. TWH_ExQ, time spent walking and time doing housework by Exernet questionnaire; TWH_MLTPAQ, time spent walking and time doing housework by Minnesota questionnaire; TPA_ExQ, time performing regular physical activity by Exernet questionnaire;

TPA_MLTPAQ, time performing regular physical activity by Minnesota questionnaire; TSS, time spent sitting by Exernet questionnaire.

Each PF test was compared between the four groups: inactive and high sedentary, inactive and low sedentary, active and high sedentary and active and low sedentary controlling by sex, respectively (Figures 6-13). In males, there were significant differences between inactive and high sedentary and active and low sedentary ($p<0.05$) groups for the 8-foot up-and-go test. Also, there were significant differences between inactive and low sedentary and active and low sedentary, and between inactive and high sedentary and active and low sedentary for the 6-min walk test ($p<0.001$) and chair stand test ($p<0.05$). In contrast, no significant differences were found in females.

^aSignificant differences between inactive and high sedentary and active and low sedentary. * Level set at $p<0.05$. The statistical analyses were made adjusting for age.

Figure 6. 8-foot up-and-go test by groups: inactive and high sedentary, inactive and low sedentary, active and high sedentary and active and low sedentary in males.

^{a,b}significant differences between inactive and high sedentary and active and low sedentary, also for inactive and low sedentary and active and low sedentary. *** Level set at $p < 0.001$. The statistical analyses were made adjusting for age.

Figure 7. 6-min walk test by groups: inactive and high sedentary, inactive and low sedentary, active and high sedentary and active and low sedentary in males.

Level set at $p < 0.05$. The statistical analyses were made adjusting for age.

Figure 8. Handgrip strength test by groups: inactive and high sedentary, inactive and low sedentary, active and high sedentary and active and low sedentary in males.

^{a,b} Significant differences between inactive and high sedentary and active and low sedentary, also for inactive and low sedentary and active and low sedentary. * Level set at $p < 0.05$. The statistical analyses were made adjusting for age.

Figure 9. Chair stand test by groups: inactive and high sedentary, inactive and low sedentary, active and high sedentary and active and low sedentary in males.

Level set at $p < 0.05$. The statistical analyses were made adjusting for age.

Figure 10. 8-foot up-and-go by groups: inactive and high sedentary, inactive and low sedentary, active and high sedentary and active and low sedentary in females.

Level set at $p < 0.05$. The statistical analyses were made adjusting for age.

Figure 11. 6 min walk test by groups: inactive and high sedentary, inactive and low sedentary, active and high sedentary and active and low sedentary in females.

Level set at $p < 0.05$. The statistical analyses were made adjusting for age.

Figure 12. Handgrip strength test by groups: inactive and high sedentary, inactive and low sedentary, active and high sedentary and active and low sedentary in females.

Level set at $p < 0.05$. The statistical analyses were made adjusting for age.

Figure 13. Chair stand test by groups: inactive and high sedentary, inactive and low sedentary, active and high sedentary and active and low sedentary in females.

4.2.5 Discussion

The results from this cross-sectional study indicate that the worst aerobic endurance ($p < 0.001$) and lower body strength ($p < 0.05$) were obtained in males from both inactive groups, independently of sedentary behaviour. Agility was highest in the active and low sedentary group ($p < 0.05$). In our study, significant differences were not found in the new classification and PF tests in females as they presented lower PF performance (51). Likewise, PA and sedentary behaviour classification showed a correlation with PF tests in males, but does not discriminate for handgrip strength in males and in all PF tests in females.

The importance of PF in maintaining overall health with advancing age is well established (178,290). However, most studies assessing PA with questionnaires for methodological or economic reasons. The different protocols used to measure PA in the elderly population might explain the dissimilar results of these investigations (20). The personal response of each participant about the time consumed for any activity depends on the kind of intensity. For instance, in participants who claimed to play tennis for one hour, this response was probably true. Nevertheless, if a participant reports one hour of work at home, probably he/she overestimates this duration as this activity is often

habitual. Biswas et al. suggested that as there were several definitions and quantitative cut-offs for PA there is a need to establish the same criteria (39). Bermudez et al. performed a study on overestimation of PA and found that the highest median was in household activities followed by occupation, transportation and leisure time activities using International Physical Activity Questionnaire (34). We observed this phenomenon when working with MLTPAQ and ExQ to ascertain TPA and TWH durations. The mean of declared durations of TPA for males (52 min/day) was less than for TWH (109 min/day). This pattern was also found in females, for TPA and TWH, with 32 min/day and 136 min/day, respectively. A combination of both questionnaires has been used since a significant correlation ($p < 0.05$) between them was found for TPA ($r = 0.63$) and TWH ($r = 0.55$), in order to reduce errors common to self-reported data.

Housework activities may increase overall activity levels, but these activities are not intensive enough to achieve the moderate-to-vigorous intensity recommended for optimal health (328). The findings on housework activities and health are mixed, with several studies finding that housework activities are unrelated or positively related to obesity (216,336) and others finding associations with reduced risk of cardiovascular and all-cause mortality (2,242).

Surprisingly, while it is well established that sedentary behaviour is associated with negative health (147,350), sedentary behaviour has not been included in most questionnaires until recently or is still not included. There is no universal consensus on the definition of sedentary behaviour. Jefferis et al. used < 100 counts per minute (cpm), while other authors suggested lower cut-offs of 25 cpm and 50 cpm (167). Salavane et al. have defined sedentary behaviour as follows: > 2 h watching TV daily, screen time > 3 h daily or ≥ 6 h on weekdays (307). In our study, 69.9% of males and 57.1% of females spent more than three hours in sedentary behaviour according to the estimation proposed by Eaton (94).

Diverse studies have shown an independent association between sedentary behaviour and PA but not all the authors are in agreement (39,219). In this study, we observed a similar pattern since TSS was independent of duration of PA, both in males and females. Morales et al. found that sedentary time has negative influence on PF among postmenopausal females independently of walking time (234). The usefulness of the physical tests mentioned above compared to PA questionnaires is their objective nature. But to obtain a holistic approach to the reality of PA patterns, we believe that the

combination of PA with TSS, as proposed here, could be an adequate method to be taken into account to establish a behaviour pattern for the population. Accurate quantification of PA and PF becomes essential in terms of health outcomes and effectiveness of intervention programs (203). Clinical practice should recommend lifestyle changes resulting in a combination of increased PA and PF and reduced TSS, most likely resulting in positive health changes.

Limitations and strengths

This study has several limitations. Firstly, our study is directly related to the intrinsic nature of ExQ, in which only bounded answers were available for several questions and also to the participantivity of the PA and sedentary questionnaires. This aspect could report errors and bias. Secondly, this study had a cross-sectional design, thus impeding the determination of cause-effect relationships. Thirdly, the sample consisted of people who participated voluntarily and they were recruited in different sites (i.e. sport federations, sports facilities, municipal clubs and health centres), so this could address some bias. On the other hand, this study also has several strengths. One of the strengths of our study was the use of clusters. Clustering of activities produces an alternative approach to summarizing PA participation and may provide a helpful methodological development when questionnaires are used to assess PA. Another strength is the inclusion of a battery of PF tests, a more objective and precise method than questionnaires, and furthermore, the combination of both methods to classify our population allowed us to have a realistic and wide vision about their behaviour pattern. Moreover, the sampling procedure and the strict standardization of the field work among the cities involved in the study avoided confounding bias to a great extent.

4.2.6 Conclusions

The proposed classification is valid as it classifies participants according to their PA and sedentary behaviour and outcomes are related to fitness measures, especially in males. When analysed separately, physical activity/inactivity is more strongly associated with fitness than high and low sedentary behaviour in older people, but only significantly in males. Further studies are necessary specifically in females due to the results obtained. This study proposes a practical approach that should allow public health authorities,

researchers and physicians to work with a novel classification concerning physical activity and sedentarism.

4.3 ARTICLE 3: IMPACT OF PHYSICAL ACTIVITY AND SEDENTARISM ON HYDRATION STATUS AND LIQUID INTAKE IN SPANISH OLDER ADULTS. THE PHYSMED STUDY

Impact of physical activity and sedentarism on hydration status and liquid intake in Spanish older adults. The PHYSMED study

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4.3.1 Abstract

Data on hydration status in older adults are scarce and there are very few studies focusing on the impact of physical activity (PA) and physical fitness (PF) on drinking behaviour. To assess the impact of PA and sedentarism and PF on fluid intake in Spanish old adults. **Methods:** 433 non-institutionalized Spanish older adults (57% females), aged 55-88 yr volunteered for the PHYSMED study. PA data were obtained by means of the Minnesota and EXERNET questionnaires. Population was divided into four groups: ILS (inactive and low sedentary), IHS (inactive and high sedentary), ALS (active and low sedentary) and AHS (active and high sedentary). Furthermore, PF was performed through a battery of PF tests and participants were divided into three levels: low, medium and high PF. Serum from fasting blood samples was analysed for osmolarity. **Results:** The mean of total liquid intake was 1751 mL/day. Significant differences were observed for total liquid intake between ILS/ALS and IHS/ALS ($p < 0.001$). There was a significant difference for water intake between PA and sedentary groups ($p < 0.01$). ALS participants consumed higher amount of beverages such as water, juice, milk, coffee, sport drink, beer, wine and distilled drinks than the other groups; however, different patterns were observed according to PF level. Only a significant difference between distilled drinks and PF level were found. Serum osmolarity values were within references ranges in all participants, and there was a

significant difference between PA and sedentary groups ($p < 0.01$). **Conclusions:** Spanish older adults meet the DACH recommendations set by the German, Austrian and Swiss nutrition societies liquid intake recommendations in the mean independently of PA and sedentary groups and PF level. All participants are within reference ranges of serum osmolarity. Participants in the active and low sedentary group consumed higher amounts of water and other beverages than in the other groups.

Key words: beverages; water intake; physical activity; elderly; sedentary lifestyle.

4.3.2 Introduction

Quantification of water and beverage intake is an emerging topic in nutritional sciences as the optimal functioning of our body requires a proper hydration level (121,170). Likewise, water intake and hydration status have recently gained attention as one of the many, and potentially manageable factors associated with disease development and wellbeing (63). Water is an essential nutrient for human body and major key to survival (268).

However, the current lack of a hydration assessment gold standard greatly impedes attempts to link water intake and negative health outcomes as well as to make public dietary guidelines (214,240). The questions „what do you drink“ and „how much do you drink per day“ „how much fluids do you drink“ are frequently omitted in the common dietary questionnaires, and recommendations for beverage intake are or were until recently, missing in most „food pyramids“ (135,184).

Maintaining an adequate fluid balance is an essential component of health at every stage of life, especially in elderly population since older adults are at higher risk of developing dehydration for various physiological reasons (184). Moreover, in elderly individuals, adequate fluid consumption has been associated with fewer falls, lower rates of constipation and laxative use, as well as better rehabilitation outcomes in orthopaedic patients (224,225,323). Also, dehydration can precipitate emergency hospitalization and increases the risk of repeated hospitalizations (294).

Elderly may frequently meet difficulties in gaining access to beverages due to decreased mobility, visual problems, swallowing disorders, cognitive alterations, use of drugs and fear of incontinence (184). Numerous factors, such as, high ambient temperature and humidity levels, heat stress and physical activity (PA) can influence water needs (315). Therefore, adequate intake of fluids must be increased in relation to these conditions (170). High temperature and humidity also might provoke exacerbated dehydration, but there is limited information about the effects of seasonality and climate on water intake (369).

The benefits of regular PA on health and disease prevention are well recognized (245). PA reduces both morbidity and mortality and can minimize the physiological effects of an otherwise sedentary lifestyle increasing the active lifespan (39,219). On the other hand, prolonged sitting has emerged as a risk factor for early mortality and has deleterious health effects. Nevertheless, sedentarism has not been included in the past in

studies about PA (39). In spite of the powerful benefits of PA, physical fitness (PF) and optimal hydration status, there are only a few studies analysing together fluid intake, PA, sedentarism and PF in older population. Hence, these parameters are a key point to define public health recommendations.

The aims of this study were (1) to assess the impact of PA and sedentarism on fluid intake in Spanish older adults (2) an additional evaluation of the effect of PF on water and beverage intake in Spanish older adults.

4.3.3 Material and Methods

Study design, sample and ethics

The present study was based on a cross-sectional multicentre study aiming at identifying cardiovascular risk factors in sedentary and active old participants. The survey was conducted from April 2013 to May 2014 in Madrid and Mallorca (Spain). Participants were voluntaries recruited at health centres, sport federations, sport facilities and municipal clubs located at Madrid and Mallorca through a snowball system. A total of 433 participants, 186 male (43%) and 247 females (57%) aged 55 to 88 yr old were included in this study. The exclusion criteria were age under 55 yr, being institutionalized, suffering from a physical or mental illness which would have limited their participation in the PF tests or their ability to respond to the questionnaires, or drug intake for clinical research. All measurements at each institution were conducted according to the survey protocol.

The study was performed according to the principles established in the Declaration of Helsinki and approved by the Ethical Committee of the Technical University of Madrid. Written informed consent was obtained from all participants.

Hydration questionnaire

A hydration questionnaire was developed by the Research Group (213) based on food-frequency and eating habits questionnaires published in the literature (99,239), taking into account the modern beverage market.

The questionnaire is divided into two parts. The first part includes questions about the different types of fluids consumed one day before the questionnaire was filled out. The second part records the fluid intake during a normal week for each beverage type. The

beverages included were: water, juices, soft drinks, diet drinks, milk, shake, coffee, tea or infusions, sport drinks, beer, wine and distilled drinks.

Physical activity and sedentarism assessment

PA was assessed by means of two validated self-reported questionnaires; the Minnesota Leisure Time Physical Activity Questionnaire (MLTPAQ) measuring the activity during the last year, and the EXERNET questionnaire (ExQ), both of them validated for the Spanish population (96,97,202).

The MLTPAQ includes diverse physical activities and participants are asked about PA duration, weekly and monthly frequencies. Time for each activity was expressed in minutes per day (min/day). On the other hand, the ExQ included four questions about regular PA duration, time spent walking, time spent doing housework and time spent sitting (TSS) per day. All questions were bounded and answers included six options which were classified as less than 1 h, between 1 and 2 h, between 2 and 3 h, between 3 and 4 h, between 4 and 5 h and more than 5 h. The only exception was for regular PA duration. All activities were recorded in min/day.

In order to compare between different levels of PA and sedentarism, our population was classified in 4 groups: inactive and low sedentary (ILS), inactive and high sedentary (IHS), active and low sedentary (ALS) and active and high sedentary (AHS) taking into account cluster analyses set by Aparicio-Ugarriza R et al. (22).

Physical fitness tests

Prior to assess PF tests, blood pressure was measure using digital monitor (Omron Healthcare Co., Ltd. Kyoto, Japan). Each participant completed a multi-component battery of PF test validated in the elderly population (290) and validated reference ranges for Spanish older adults proposed by Pedrero-Chamizo et al. (269). Lower body strength was measured by the chair stand test (290), agility/dynamic balance was performed by the 8-foot up-and-go test (290), aerobic endurance was assessed by the 6-min walk test (290) and handgrip strength was measured with a handgrip dynamometer (Takei TTK 5401, Tokyo, Japan, range 5-100 kg, precision 0.1 kg) (361). The handgrip strength was assessed for both hands in a standing position. All tests were performed twice, except the 6-min walk test and the chair stand test, and the best score was retained.

The results of each PF tests were stratified by sex and age groups (distributed by five years periods without truncating the last group) following the criteria established by Pedrero-Chamizo et al. (269). Also, the result of each PF test was divided into quartiles considering the variables above mentioned (sex and age groups). The score for each test ranged from 0 (worst) to 3 (best) points. Thus, the maximum score was 12 points. Scores of PF tests were added together to create a cluster. After that, PF cluster analyses were performed and each cluster was divided into different levels. In order to classify our population taking into account analysis of clustering, we established three groups of PF: low, medium and high.

Serum osmolarity

Fasting blood samples were collected from each participant by standard venepuncture on vacuum Vacutainer® tubes with separation gel in Madrid and Palma de Mallorca, at the biochemical laboratory of the High Sports Council, Madrid, Son Espases Hospital and University of the Balearic Islands, Palma de Mallorca, respectively. Serum was separated in 1 mL eppendorfs and was processed to analyse osmolarity using an osmometer Osmo Station OM-6050 (Menarini Diagnostics, Florence, Italy, CV≤1%).

Statistical analysis

Descriptive values are shown as mean±standard deviation (SD) unless otherwise stated. One-way ANOVA was performed to analyse the differences between serum osmolarity, total liquid intake and beverages consumption and *post hoc* analyses were conducted with Bonferroni adjustment according to the PA and sedentarism groups (ALS, AHS, IHS and ILS) and PF level (low, medium and high).

All analysis was performed using the Statistical Package for Social Science software (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp) and values of $p<0.05$ were considered statistically significant.

4.3.4 Results

Table 17 includes descriptive characteristics of the sample split by sex. Males were heavier and taller than females ($p<0.01$) and had higher BMI ($p<0.05$). No differences by sex were observed for mean serum osmolarity.

Table 17. Descriptive characteristics of the study sample.

	Male (n=186)	Female (n=247)	p
Age (yr)*	65.4±6.6	67.5±6.6	<0.01
Height (cm)*	170.2±6.6	156.6±5.6	<0.01
Weight (kg)*	79.9±10.8	65.4±10.4	<0.01
BMI (kg/m ²)*	27.5±3.1	26.7±4.3	<0.05
City†			
Madrid	78 (49.1)	122 (49.4)	
Mallorca	108 (58.1)	125 (50.6)	>0.05
Serum osmolarity (mOsm/L)*	289.3±5.3	289.2±4.5	>0.05

Results are expressed as follows: *mean ± SD; †n (%). Significant differences between sex by one way ANOVA test. Level of significance p<0.05.

Figure 14 shows serum osmolarity according to PA and sedentary groups. All participants were within reference ranges of serum osmolarity and significant differences were found between PA and sedentary groups (p<0.01). After Bonferroni's adjustment, there was a significant difference between IHS and ALS (p<0.05). The mean higher serum osmolarity was obtained for ALS group (291.0 mOsm/L). Nevertheless, there were not observed significant differences between serum osmolarity and PF levels (Figure 15).

Horizontal lines represent serum osmolarity reference ranges limits. ^{a*}, significant difference (level set at p<0.05) between inactive and high sedentary and active and low sedentary after Bonferroni's adjustment.

Figure 14. Serum osmolarity divided by physical activity groups.

Horizontal lines represent serum osmolality reference ranges limits. Level of significance $p < 0.05$.

Figure 15. Serum osmolality split by physical fitness groups.

Figure 16 shows the mean ($\pm$ SD) total liquid intake per day consumed according to PA and sedentary groups. ALS group and AHS group consumed 2056 ± 679.6 mL/day and 1900 ± 633.4 mL/day, respectively. On the other hand, the mean of total liquid intake was 1648 ± 569.4 mL/day for ILS group and 1648 ± 597.2 mL/day for IHS group. There were significant differences between ALS and ILS groups and also between ALS and IHS groups ($p < 0.001$). There were not found significant differences for total fluid intake according to PF levels.

***a,b Significant differences (level set at $p < 0.001$) between inactive and high sedentary and active and low sedentary.

Figure 16. Mean ($\pm$ SD) total liquid intake/day (mL/day) according to physical activity groups.

Level of significance $p < 0.05$.

Figure 17. Mean ($\pm$ SD) total liquid intake/day (mL/day) according to physical fitness groups.

Figure 18 shows the mean beverage consumptions per day divided by PA and sedentary groups. Water was the beverage most consumed for all PA and sedentary groups and there were significant differences between them ($p < 0.01$). ALS drank more beverages

such as water, juice, milk, coffee, sport drink, beer, wine and distilled drinks than the other PA and sedentary groups. Significant difference was only observed for water between PA and sedentary groups ($p < 0.01$). Moreover, AHS showed a trend to consume more soft and light soft drinks, shake and tea than the other PA and sedentary groups (non-significant values).

The only significant difference was among medium and high PF group for distilled drink ($p < 0.05$). High PF groups showed higher intake of juice, light soft drink, shake, tea, sport drink and beer than medium and lower groups (Figure 19).

**aSignificant differences ($p < 0.01$) between physical activity and sedentary groups for water.

Figure 18. Mean beverage consumptions (mL/day) divided by physical activity groups.

*Significant difference between physical fitness groups and distilled drink. Level of significance $p < 0.05$.

Figure 19. Mean beverage consumptions (mL/day) divided by physical fitness groups.

4.3.5 Discussion

The results from this cross-sectional study indicated that the mean of total liquid intake in our sample was high (1751 mL/day, data are not shown) compared with the German, Austrian and Swiss nutrition societies (DACH) recommendations (1310 mL/day) (86). ALS group consumed significantly higher amounts of water and showed a trend of high consumption of other fluids than the other PA and sedentary groups. Nevertheless, there were not significant differences between fluid intake and PF level, except for distilled drinks. Significant differences in serum osmolality between PA and sedentary groups were found. However, despite these differences, all participants were within serum osmolality reference ranges.

There is no consensus in the literature regarding water and beverage intake recommendations. We compared to the DACH recommendations because they split recommendations into water from beverages and from food specifically for people aged over 65 yr (86). In contrast, the World Health Organization in 2005 recommended 2.2

L/day and 2.9 L/day for females and males, respectively (370). The European Food Safety Authority in 2010 established 2 L/day for females and 2.5 L/day for males for total water intake without differentiating into water from food and beverages (100). Other author considers that the minimum water intake for drinking water and beverages must range from 1.5-1.8L/day; although some adaptations are done depending on age, sex and medications (184).

Likewise, the best way of measuring hydration in humans is still a general controversy (248) because there are different methods such as estimates of water balance (thirst rating, total water intake and output or body weight changes), hydration markers (plasma or urine osmolality) and total body water measurements by bioelectrical impedance or isotope dilution (229). In our study, we measured serum osmolality due to the fact that it is closely controlled and rarely varies by more than 2% around a set point of 280-290 mosm/L (170). According to Jequier et al., a basal mean value of 287 mOsm/L is maintained in well-hydrated individuals (170). In our sample, the mean of serum osmolality levels was 289 mOsm/L in both sexes though a significant difference was obtained between PA groups. Nevertheless, serum osmolality did not show significant difference with PF groups.

On the other hand, in our study, water was the beverage most consumption in all PA and sedentary groups and a significant difference was obtained between them followed by light soft drinks. In their study, Zizza et al. found that water was the most consumption followed by coffee (386). De Francisco et al. observed that people with high levels of PA drank more fluids compared with those with low PA levels (10). We found the same patterns in our study since both active groups (ALS and AHS) had higher intakes than both inactive groups. On the other hand, medium PF group showed a higher intake of total liquid intake than in the low PF group. Andrianopoulou et al. showed that dehydrate people had significant lower scored in 6 walking minutes test and chair sit-to-stand (17).

Furthermore, we found that ALS participants drank higher amounts of water, juice, milk, coffee, sport drinks, beer, wine and distilled drinks than the other PA and sedentary groups. Additionally, AHS consumed more soft and light soft drinks, milk shakes and tea than the other PA and sedentary groups. Regarding PF level, high PF group showed higher intake of juice, light soft drink, shake, tea, sport drink and beer than the other PF groups.

This study has several strengths. One of them was the use of clusters. Clustering of activities produced an alternative approach to summarizing PA participation and may provide a helpful methodological development when questionnaires are used to assess PA and sedentary behaviour. Likewise, data on hydration status has been analysed through a battery of validated PF tests. An additional strength was the use of a specific hydration questionnaire in order to obtain reliable data on water and beverage intake. Moreover, the sampling procedure and the strict standardization of the field work among the cities involved in the study avoided to a great extent the kind of confounding bias.

On the other hand, this study has also several limitations. First, it is directly related to the intrinsic nature of the ExQ, in which for several questions only closed answers were available and also for the participantiveness of the PA, sedentary behaviour and hydration questionnaires. Secondly, our study has a cross-sectional design, therefore impeding the determination of cause-effect relationships.

4.3.6 Conclusion

The mean of Spanish older adults meets DACH liquid intake recommendations independently of PA, sedentary behaviour and PF level; furthermore, all participants are within reference ranges of serum osmolarity. Participants in the active and low sedentary group consumed higher amounts of water and other beverages than in the other PA and sedentary groups. A significant difference between PF level and distilled drinks were observed. PA, sedentarism and PF should be considered to obtain a holistic approach to beverage intake and hydration status in future researches. Additionally, longitudinal studies are needed in order to establish behaviour patterns.

4.4 ARTICLE 4: WHAT IS THE RELATIONSHIP BETWEEN PHYSICAL FITNESS LEVEL AND MACRO AND MICRONUTRIENTS INTAKE IN SPANISH OLDER ADULTS?

Aparicio-Ugarriza R, Luzardo-Socorro R, Palacios G, Bibiloni MM, Argelich E, Tur JA, González-Gross M. Eur J Nutr (submitted). (JCR: 4.370).

4.4.1 Abstract

Nutrition and physical fitness (PF) modifications seem to be strong promoters of healthy ageing. The aim of this study was to assess the association between PF and energy and nutrient intake in Spanish older adults. **Methods:** 324 participants (59.9% females, aged over 55 yr) performed a battery of 4 validated PF tests and participants were divided into 3 PF groups: low, medium and high. Dietary intake was assessed by two non-consecutive 24h dietary recalls. Energy and nutrient intake were calculated using ALIMENTA software. Energy expenditure (EE) was calculated through physical activity questionnaire using METs min/week. **Results:** The mean energy intake (EI) was 2123 kcal/day, 2066 kcal/day and 2209 kcal/day in the low, medium and high PF in males, respectively. In females, the EI was 1647 kcal/day, 1608 kcal/day and 1665 kcal/day in the low, medium and high PF group. For all macronutrients, only a positive association was observed for vegetable proteins ($p<0.05$). There were significant and positive associations between high PF group and phosphorous, selenium, vitamin B₆, vitamin C, vitamin D, vitamin E, niacin and folates (all $p<0.05$). However, high PF group presented negative associations with thiamine and riboflavin (all $p<0.05$). A total of 8.3% of participants presented inadequate intake of 11 micronutrients. **Conclusions:** PF seems to affect total nutrient intake. Higher macronutrients intake were observed in the high PF group compared to the other PF groups, but participants in the high PF group had also higher EE, especially in males. Participants in the high PF group showed a better micronutrient intake profile than the others PF groups. Also, there are a significant number of participants who presented inadequate intake of micronutrients. There is a requirement to develop nutritional programs and fitness among older adults and to consider supplementation for micronutrients at risk.

Key words: physical fitness; macronutrients; vitamins; minerals; diet records; aging.

4.4.2 Introduction

The role of physical fitness (PF) and diet in reducing the progression of chronic disease is becoming increasingly crucial (126). PF and diet have been described as major modifiable behaviours related to poor health (58). Some authors suggest that improvements in these factors could prevent the functional limitations that are strongly associated with advanced age, and could lead to a healthier, more active, and more independent way of aging (293,342).

Older adults are particularly at risk of deficient or suboptimal micronutrient status (136,233). As energy expenditure (EE) decreases in the older population (209), mainly because of less daily activity (368), a reduction of food intake leading to a proportional reduction of the intake of some nutrients is often the consequence (296). Deficiencies in micronutrient intake increase the disease risk and several vitamins and minerals intake deficiency has been observed previously in older adults (316). Meanwhile, the prevalence of malnutrition is increasing in this kind of population (4), which is associated with a decline in functional status (104), impaired muscle function and sarcopenia (92), anaemia (295), immune dysfunction (117), decreased bone mass and reduced cognitive function (4).

PF is accepted as a health marker and is also considered as an independent factor of all cause-diseases and mortality (43,269). A 50% of reduction in mortality has been observed among highly fit people compared to low fit people (226). Additionally, PF is dependent on several factors like nutritional habits, smoking habits, genetics and socio-economic status (141).

Because the number of elderly is increasing in developed countries, it is essential to understand the effects of modifiable risk factors such as PF and diet (141). There are few data analysing the association between PF level and diet in Spanish old population (215). Nevertheless, both PF and diet is a key for the development of public health policies to promote healthy lifestyle based on the evidence (321). PF and diet can keep adults and elderly healthy, fit and independent (226). Therefore, the aim of this study was to investigate the relationship between energy and nutrient intake with PF level in Spanish older adults.

4.4.3 *Materials and Methods*

Study design, sample and ethics

The present study was based on a cross-sectional multi-centre study aiming at identifying cardiovascular risk factors in active and sedentary old participants. Data collection took place from April 2013 to May 2014 in Madrid and Mallorca (Spain). Volunteers were recruited through health centres, sport federations, sport facilities and municipal clubs located at Madrid and Mallorca by snowball system. The study population consisted of 433 participants, 184 male (43%) and 244 females (57%) aged 55 to 88 yr. The exclusion criteria were age under 55 yr, being institutionalized, suffering from a physical or mental illness which would have limited their participation in the PF tests or their ability to respond to the questionnaires or drug intake for clinical research. For the purpose of this study, participants who provided data on two non-consecutive 24h dietary recalls were included in the analysis, resulting in 428 older adults. Likewise, underreporting was considered when the individual ratio of energy intake (EI) divided by the estimated basal metabolic rate was lower than 0.96 (EI/basal metabolic rate <0.96) (41). Excluding the underreporters, valid reporters resulted in a final sample of 324 participants (59.9% females) for statistical analysis. The group of underreporters consisted of slightly higher percentage of females (57.0% compared to 59.9%, $p=0.035$) and had higher median body mass index (BMI) values (28.2 kg/m^2 compared to 26.5 kg/m^2), weight (77.0 kg compared to 68.4 kg) and fat mass (24.6 kg compared to 21.4 kg) (all $p<0.001$). No difference in age was observed. The number of participants in each group of PF considering all sample and underreporting, and macro and micronutrient in underreporters participants are shown at the end of this Article (Annex 1-2).

The study was performed according to the principles established in the Declaration of Helsinki and the final protocol was approved by the Ethical Committee of the Technical University of Madrid. All participants were informed of the protocol and older adults signed a written informed consent prior to participation.

Physical activity assessment

The Minnesota Leisure Time Physical Activity Questionnaire was carried out by trained interviewers. It has been validated for Spanish males (97) and females (96). In order to calculate the total energy expenditure of physical activity (PA), an intensity value was

assigned to all the items in the PA questionnaire, according to the METs classification proposed by Ainsworth (5). Total PA was calculated by multiplying the total number of hours and their correspondent intensity performed for each activity.

Physical fitness tests

Each participant completed a multi-component battery of PF tests (290) and validated reference ranges for Spanish older adults proposed by Pedrero-Chamizo et al. (269). Lower body strength was measured by the chair stand test (290), agility/dynamic balance by the 8-foot up-and-go test (290), aerobic endurance by the 6-min walk test (290) and handgrip strength with a dynamometer (Takei TKK-5401, Tokyo, Japan, range 5-100 kg, precision 0.1 kg) (361). The handgrip strength was assessed for both hands in a standing position. All tests were performed twice, except the 6-min walk test and the chair stand test, and the best score was retained.

The results of each PF tests were stratified by sex and age groups (distributed by five years periods without truncating the last group) as previously proposed by Pedrero-Chamizo et al. (269). Also, the result of each PF test was divided into quartiles considering the variables above mentioned (sex and age groups). The score for each test ranged from 0 (worst) to 3 (best) points. Thus, the maximum score was 12 points. Scores of PF tests were added together to create a cluster. After that, PF cluster analyses were performed and each cluster was divided into different levels. In order to classify our population taking into account analysis of clustering, we established three groups of PF: low, medium and high.

Anthropometric measurements

Height was measured to the nearest millimetre using a mobile stadiometer (SECA 213, Germany), with the participant's head in the Frankfurt plane. Weight and body composition (fat mass and fat free mass) was assessed by means of bioimpedance analysis (TANITA Corp, BC-418MA, Tokyo, Japan) in standardized conditions. Also, BMI was calculated as weight ($\text{kg}/\text{height}^2$) (kg/m^2). Waist and hip circumference were performed by a trained ISAK level II anthropometrist in order to minimize the inter-observer coefficients of variation according to ISAK Society (210).

Dietary intake assessment

Dietary intake was obtained by means of two 24h dietary recalls by well-trained dietitians. The 24h dietary recalls were collected on two non-consecutive days within a period of 2-3 weeks. Volumes and portion sizes were reported with the aid of a book of photographs (130). Energy and nutrient intake were calculated using a computer program (ALIMENTA; NUCOX, Palma, Spain) based on Spanish (218,255) and European (106) food composition tables, and complemented with food composition data available for Mallorca food items (291).

Furthermore, the estimated average requirements (EAR) cut-off point was applied to estimate the prevalence of nutrient intake adequacy (162). When EAR was not defined for a specific micronutrient, such as pantothenic acid, potassium and sodium, the adequate intake (AI) was chosen (162).

Statistical analysis

Statistical analyses were run on SPSS (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp). Descriptive characteristics were summarized by calculating means and standard deviations (SD) unless otherwise stated. All PF test and dietary intakes were checked for normality of distribution with the Kolmogorov-Smirnov test. Macro and micronutrient results were transformed (log-transformation) due to showed non-normally distribution except for energy, water, fat and mono-unsaturated fatty acids. Comparisons between PF groups and sexes with nutrient intake were analysed by One Way ANOVA or Kruskal-Wallis test, according to the normality of the variables.

In order to determinate the adequacy or inadequacies of nutrient intake, participants were split into two groups taking into account EAR or AI: below EAR or AI (inadequate; coded as 0) and above EAR or AI (adequate; coded as 1).

Multilevel linear regression analysis was used to examine the relationship between the macro and micronutrient intake, energy expenditure and PF groups. Confounders (age and sex) were entered as covariates. To adjust for multiple testing, a Bonferroni correction was applied to lower the significance level (α) taking into account the number of tests ($0.05/\text{number of tests}$). A p-value of 0.0013 was used as threshold of significance for the association between macro and micronutrient intake, energy expenditure and PF groups.

4.4.4 Results

Table 18 shows descriptive characteristics of the sample divided by sex. Table 19 displays energy expenditure, macronutrients and micronutrients intake split by sex and PF groups. Energy expenditure, macro and micronutrient for underreporters are shown in Annex 2 (at the end of this Article).

Table 18. Descriptive characteristics of the sample split by sex.

Variables	Male (n=130)	Female (n=194)
	Mean±SD Median (min-max)	Mean±SD Median (min-max)
Age (yr)	64.9±6.5 64.2 (55.0-80.0)	67.2±6.7 67.0 (55.1-87.8)
Weight (kg)	78.7±11.1 78.3 (44.9-116.6)	63.8±9.7 63.5 (44.0-97.9)
Height (cm)	169.8±6.6 169.9 (151.7-185.5)	156.6±5.6 156.5 (142.0-176.2)
BMI (kg/m ²)	27.2±3.2 27.3 (17.1-37.6)	26.1±4.0 25.8 (17.0-41.8)
Fat mass (kg)	20.7±6.1 20.5 (6.4-39.9)	23.2±7.1 22.7 (7.3-48.7)
Fat free mass (kg)	58.0±6.7 58.0 (37.3-76.7)	40.6±3.8 40.2 (33.7-55.1)
Waist circumference (cm)	95.3±10.4 96.0 (67.0-124.7)	82.8±9.5 82.2 (61.3-111.7)
Hip circumference (cm)	99.8±6.0 100.0 (85.7-119.0)	100.9±8.4 100.5 (82.7-136.8)

Data are presented as mean±SD and median (min-max). BMI, body mass index.

Table 19. Energy expenditure, macronutrients and micronutrients intake split by sex and physical fitness groups.

	Male			Female		
	Low fitness (n=30)	Medium fitness (n=59)	High fitness (n=41)	Low fitness (n=37)	Medium fitness (n=95)	High fitness (n=62)
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
	Median (min-max)	Median (min-max)	Median (min-max)	Median (min-max)	Median (min-max)	Median (min-max)
Energy expenditure (METs min/week)	3863±2636 3485 (228.4-9776)	5111±3113 4607 (528-14935)	5205±2560 4901 (1178-10963)	4230±2482 4142 (420.0-11420)	4391±2132 4189 (418.2-11162)	4398±2015 3966 (157.3-10013)
Macronutrients						
Energy (Kcal/day)	2123±379.0 2135 (1436-3121)	2066±378.2 1999 (1445-3154)	2209±418.2 2111 (1555-3514)	1647±311.7 1576 (1125-2725)	1608±310.2 1564 (1032-2877)	1665±365.3 1625 (1076-2996)
Water (ml/day)	2481±777.7 2382 (1079-4210)	2389±676.8 2223 (1344-4568)	2390±664.1 2434 (1117-3400)	2184±676.3 2029 (1324-4382)	2186±636.3 2200 (979.2-4022)	2143±618.5 2062 (637.0-4258)
Proteins (g/day) †	85.1±31.8 78.0 (43.6-179.8)	81.6±20.2 76.2 (48.1-140.9)	90.8±33.7 85.8 (47.7-242.9)	67.7±15.9 69.6 (40.6-92.1)	66.3±19.0 64.3 (30.3-141.0)	68.2±20.9 64.9 (39.9-162.6)
Proteins (% E)	15.8	15.9	16.3	16.6	16.6	16.3
Vegetable protein (g/day) †	22.7±6.9 22.8 (10.5-38.3)	22.7±7.3 22.0 (12.0-48.7)	26.6±8.9 25.8 (12.4-46.3)	20.0±5.9 21.1 (8.8-33.4)	19.3±6.8 18.6 (8.5-47.3)	20.9±6.6 20.5 (8.7-41.5)
Animal protein (g/day) †	56.6±31.3 47.9 (20.8-152.7)	54.3±19.0 50.4 (3.7-102.0)	61.2±32.8 54.5 (23.8-209.8)	43.9±17.3 43.9 (18.6-75.0)	44.5±18.7 40.9 (14.9-124.6)	44.6±19.4 41.5 (20.7-133.4)
Fiber (g/day) †	18.5±6.3 17.9 (5.9-29.4)	19.9±9.6 18.5 (8.3-63.4)	22.4±8.6 21.5 (9.0-52.3)	18.1±8.9 16.3 (7.1-62.3)	17.1±5.8 16.6 (5.3-36.3)	18.1±6.1 17.4 (6.8-36.2)
Fiber (g/1000 kcal)	8.8	9.2	10.2	11.2	10.7	11.0

	Low fitness (n=30)	Medium fitness (n=59)	High fitness (n=41)	Low fitness (n=37)	Medium fitness (n=95)	High fitness (n=62)	
	Mean±SD Median (min- max)	Mean±SD Median (min- max)	Mean±SD Median (min- max)	Mean±SD Median (min- max)	Mean±SD Median (min- max)	Mean±SD Median (min- max)	p- value*
Carbohydrates							
Total carbohydrates (g/day) †	236.3±54.8 249.7 (130.6- 314.2)	224.5±57.8 212.9 (133.8- 388.3)	233.6±77.4 219.3 (100.4- 425.9)	196.4±56.7 179.9 (119.3- 361.2)	177.9±48.7 172.7 (81.8- 368.9)	191.4±47.3 189.8 (101.6- 298.6)	<0.05 a,b,c
Total carbohydrates (% E)	44.6	43.7	42.0	47.6	44.3	46.3	
Mono & disaccharides (g/day) †	104.7±30.7 107.5 (51.3-174.9)	102.8±41.2 98.9 (35.3- 215.5)	115.5±43.5 111.2 (51.4- 243.3)	91.7±30.4 88.6 (45.6- 201.1)	87.9±30.3 81.8 (24.1-175.4)	95.5±29.8 94.0 (39.4- 159.1)	<0.05 a,b,c
Polysaccharides (g/day) †^b	104.7±42.7 105.2 (22.2-174.6)	99.7±32.9 93.6 (41.2- 196.8)	107.1±53.0 94.7 (10.2- 276.3)	88.3±36.7 81.0 (29.5- 164.5)	79.7±35.1 75.1 (15.5-206.3)	86.7±40.6 80.6 (16.0- 238.7)	<0.001 a,b,c
Fat							
Fat (g/day)	80.8±14.9 81.1 (45.0-103.3)	81.2±19.5 78.7 (45.3- 126.8)	86.8±23.3 83.9 (42.1- 157.0)	61.7±18.3 61.1 (28.9- 103.9)	64.3±17.0 61.8 (26.9-105.6)	64.9±21.0 65.2 (36.4- 145.2)	<0.001 a,b,c
Fat (% E)	34.5	35.4	35.7	33.6	35.9	34.9	
SFA (g/day) †	24.9±5.7 26.2 (12.6-35.3)	23.7±8.3 21.5 (12.5-44.8)	25.4±8.7 23.7 (11.1-53.5)	18.4±7.1 18.5 (5.5-42.6)	19.1±6.4 18.7 (7.0-36.5)	19.3±9.4 18.7 (6.2-68.5)	<0.001 a,b,c
SFA (% E)	10.6	10.3	10.5	9.9	10.6	10.3	
MUFA (g/day)	35.8±8.9 37.3 (12.2-52.7)	37.2±9.6 35.8 (19.8-60.8)	39.2±11.4 39.1 (19.2-70.8)	27.9±9.4 27.4 (14.0-57.7)	29.2±8.9 28.4 (12.0-56.6)	29.3±9.5 27.9 (11.8- 67.7)	<0.001 a,b,c
MUFA (% E)	15.2	16.2	16.2	15.2	16.3	15.9	
PUFA (g/day) †	10.1±4.1 10.0 (3.4-26.9)	10.9±3.9 10.0 (5.1-22.5)	11.6±3.9 11.6 (4.3-22.9)	7.4±2.4 7.1 (3.7-13.6)	8.4±3.6 7.7 (3.2-20.9)	8.3±3.1 7.7 (3.9-16.4)	<0.001 a,b,c
PUFA (% E)	4.4	4.7	4.7	4.1	4.7	4.5	
Cholesterol (mg/day) †	353.2±218.6 308.6 (73.1-878.7)	337.9±167.1 310.7 (13.8- 912.0)	351.2±192.5 262.1 (49.7- 886.6)	238.6±119.4 229.9 (75.9- 563.2)	262.4±133.9 227.2 (73.4- 636.7)	267.2±124.9 259.5 (25.4- 649.4)	<0.05 a,b
Cholesterol (mg/1000 kcal)	165.0	163.6	156.0	144.4	163.5	163.6	

	Low fitness (n=30)	Medium fitness (n=59)	High fitness (n=41)	Low fitness (n=37)	Medium fitness (n=95)	High fitness (n=62)	
	Mean±SD Median (min- max)	Mean±SD Median (min- max)	Mean±SD Median (min- max)	Mean±SD Median (min- max)	Mean±SD Median (min- max)	Mean±SD Median (min- max)	p- value*
							p- value°
Alcohol (g/day) †	16.5±16.0 12.4 (0-50.7)	15.6±21.9 9.6 (0-141.0)	18.4±14.4 19.8 (0-50.2)	4.9±6.9 0.1 (0-26.4)	7.2±8.2 4.8 (0-37.2)	5.3±7.3 1.9 (0-29.3)	<0.05 a,b,c
Alcohol (% E)	5.4	5.0	5.9	2.1	3.0	2.0	>0.05
Minerals							
Sodium (mg/day) †	2400±1244 2095 (925.4-6068)	2136±665.1 2088 (749.9-4420)	2068±803.8 2008 (766.6-3959)	1722±613.5 1535 (966.8-3445)	1633±592.0 1552 (649.5-4399)	1660±641.8 1547 (299.7-3631)	<0.01 a,b,c
Sodium (mg/1000 kcal)	1110	1042	922.4	1047	1020	999.9	
Potassium (mg/day) †	3104±671.2 3145 (1334-4778)	3151±880.6 2902 (1804-5814)	3648±1028 3627 (2059-6443)	2732±689.0 2579 (1802-4557)	2845±727.0 2812 (13453-5582)	2976±827.9 2922 (1099-4914)	<0.05 a,c
Potassium (mg/1000 kcal)	1479	1541	1656	1698	1793	1808	
Magnesium (mg/day) †	378.98±99.9 282.6 (144.3-1691)	305.8±99.6 288.3 (160.1-642.5)	364.5±104.6 345.2 (199.4-646.0)	299.9±135.2 273.3 (157.9-793.9)	275.7±102.9 259.2 (130.5-984.0)	285.3±76.6 279.9 (130.6-509.1)	<0.05 b,c
Magnesium (mg/1000 kcal)	175.8	149.1	164.8	185.5	171.3	172.9	
Phosphorus (mg/day) †	1286±402.8 1252 (690.8-2507)	1278±311.9 1202 (807.5-2173)	1435±426.7 1324 (806.3-2905)	1075±245.6 1083 (585.3-1537)	1055±255.0 1028 (475.7-1987)	1115±341.7 1087 (511.8-2611)	<0.05 a,b,c
Phosphorous (mg/1000 kcal)	604.2	620.0	645.9	665.9	663.1	671.1	
Calcium (mg/day) †	877.9±454.3 818.8 (305.5-2843)	801.7±259.9 759.9 (473.3-1589)	846.3±297.3 761.8 (352.4-1579)	789.1±233.0 803.9 (346.5-1291)	754.7±243.9 736.6 (360.8-1595)	816.7±440.8 766.9 (215.3-3555)	>0.05
Calcium (mg/1000 kcal)	415.6	388.7	383.1	492.9	478.6	485.6	
Iron (mg/day) †	27.4±24.6 19.0 (6.4-116.7)	24.5±26.3 17.8 (8.1-197.8)	21.4±18.8 14.9 (8.9-106.6)	22.9±32.5 13.7 (6.3-199.3)	15.7±13.3 12.1 (5.8-105.7)	21.4±26.9 12.5 (5.3-154.2)	<0.05 b,c
Iron (mg/1000 kcal)	12.6	12.0	9.5	14.3	9.9	12.5	
Copper (mg/day) †	2.4±1.1 2.1 (0.9-5.5)	2.2±0.8 2.0 (0.9-4.3)	2.4±0.9 2.2 (1.1-4.3)	2.2±0.7 2.0 (0.6-3.6)	2.2±0.9 2.0 (0.6-4.7)	2.1±0.9 1.9 (0.6-5.2)	>0.05

	Low fitness (n=30)		Medium fitness (n=59)		High fitness (n=41)		Low fitness (n=37)		Medium fitness (n=95)		High fitness (n=62)	
	Mean±SD Median (min- max)		Mean±SD Median (min- max)		Mean±SD Median (min- max)		Mean±SD Median (min- max)		Mean±SD Median (min- max)		Mean±SD Median (min- max)	p- value*
Copper (mg/1000 kcal)	1.1		1.1		1.1		1.3		1.4		1.3	
Selenium (µg/day) †	114.6±56.8 97.1 (43.9-295.0)		107.5±38.4 104.5 (33.5- 202.9)		130.6±47.6 128.7 (51.4- 277.9)		85.5±31.4 86.2 (31.6- 141.1)		89.8±35.1 86.4 (38.7-266.5)		99.1±34.0 94.5 (41.9- 206.3)	<0.05 _{a,b,c} <0.01 _d
Selenium (mg/1000 kcal)	52.8		52.7		58.1		52.4		55.9		60.1	
Iodine (µg/day) †	146.8±65.1 133.6 (56.0-315.7)		138.5±53.2 134.7 (40.6- 325.1)		144.8±70.9 132.2 (56.9- 434.8)		137.1±38.9 134.8 (55.2- 231.4)		136.9±48.5 134.6 (54.1- 270.1)		128.9±41.7 122.4 (48.5- 260.1)	>0.05 >0.05
Iodine (µg /1000 kcal)	68.1		68.1		65.2		86.3		86.9		81.2	
Zinc (mg/day) †	10.5±4.1 9.6 (4.0-22.8)		12.3±14.5 9.4 (5.2-117.6)		11.0±3.4 10.5 (6.5-21.7)		9.2±3.4 8.9 (4.4-23.5)		8.7±3.8 8.0 (3.9-25.6)		10.3±7.7 8.3 (3.6-52.5)	<0.01 _{b,c} >0.05
Zinc (mg/1000 kcal)	4.9		6.2		5.0		5.7		5.4		6.1	
Vitamins												
Retinol (µg/day) †	1206±3585 311.3 (56.8- 19121)		602.6±1667 273.8 (44.4- 12607)		266.3±146.4 258.1 (71.8- 613.2)		203.9±104.4 195.3 (22.9- 427.1)		252.9±297.7 195.7 (18.8- 2428)		270.1±399.7 201.0 (22.0- 3172)	<0.01 _{a,b} >0.05
Retinol (µg /1000 kcal)	543.7		320.2		120.3		123.4		158.2		162.9	
Carotens (µg/day) †	3831±3329 2756 (368.5- 13585)		3465±2453 2997 (528.8- 10319)		4848±3719 4086 (835.5- 17286)		2932±2122 2227 (189.4- 9096)		3795±2921 2940 (359.3- 16353)		4306±3895 2717 (465.4- 18413)	>0.05 >0.05
Carotens (µg /1000 kcal)	1837.8		1842.6		2155.1		2125.3		2474.5		2559.9	
Vitamin A (ER/day) †	1098±716.4 909.5 (142.3- 2756)		1039±708.9 875.8 (176.9- 3704)		1096±635.8 944.2 (282.9- 2971)		792.8±644.5 654.3 (170.5- 4002)		911.8±568.8 762.5 (139.6- 3162)		1010±784.3 688.5 (182.6- 3962)	>0.05 >0.05
Vitamin A (ER/1000 kcal)	856.3		631.9		490.1		489.4		586.8		603.6	
Thiamin (mg/day) †	6.1±7.2 1.9 (0.5-30.6)		5.9±7.7 1.8 (0.7-32.7)		3.5±5.2 1.5 (0.7-28.7)		3.6±5.8 1.2 (0.6-29.2)		2.2±2.8 1.1 (0.5-14.2)		2.1±2.9 1.2 (0.6-19.8)	<0.05 _{b,c} >0.05
Thiamin (mg/1000 kcal)	2.8		2.8		1.5		2.1		1.4		1.2	
Riboflavin (mg/day) †	8.2±9.9 2.2 (0.9-40.4)		7.7±10.3 2.1 (0.9-42.3)		4.7±6.9 1.9 (0.7-38.3)		4.8±7.5 1.7 (0.7-36.0)		2.9±3.8 1.5 (0.6-18.9)		2.8±4.1 1.6 (0.4-26.6)	<0.05 _{b,c} >0.05

	Low fitness (n=30)	Medium fitness (n=59)	High fitness (n=41)	Low fitness (n=37)	Medium fitness (n=95)	High fitness (n=62)	
	Mean±SD Median (min- max)	Mean±SD Median (min- max)	Mean±SD Median (min- max)	Mean±SD Median (min- max)	Mean±SD Median (min- max)	Mean±SD Median (min- max)	P- value ^e
Riboflavin (mg/1000 kcal)	3.8	3.6	2.0	2.8	1.9	1.7	
Vitamin B ₆ (mg/day) †	2.0±0.8 1.8 (1.0-4.5)	2.3±1.6 1.8 (1.0-8.6)	2.7±1.9 2.3 (1.2-13.4)	2.2±2.3 1.6 (1.0-12.9)	1.8±1.1 1.6 (0.7-7.5)	2.5±2.4 1.8 (0.7-12.4)	<0.01 _{b,c}
Vitamin B ₆ (mg/1000 kcal)	0.9	1.1	1.2	1.4	1.2	1.4	
Vitamin B ₁₂ (µg/day) †	15.9±26.7 5.5 (1.8-114.3)	9.7±11.1 6.9 (0.8-76.2)	9.7±9.6 6.5 (1.8-52.1)	8.2±9.1 4.5 (1.3-38.8)	5.9±6.7 3.7 (0.8-50.3)	5.8±3.6 4.9 (1.3-16.7)	<0.01 _{b,c}
Vitamin B ₁₂ (µg/1000 kcal)	7.0	4.8	4.3	5.1	3.6	3.5	>0.05
Vitamin C (mg/day) †	136.1±68.3 141.6 (25.4-261.3)	137.0±95.7 105.7 (25.3-415.5)	181.9±103.2 163.5 (35.6-462.9)	143.6±72.8 131.7 (38.3-398.2)	148.9±82.3 133.6 (19.5-546.3)	164.6±83.6 151.1 (25.9-396.6)	<0.05 ^d
Vitamin C (mg/1000 kcal)	64.9	68.1	84.6	87.2	93.7	101.3	
Vitamin D (µg/day) †	2.4±2.9 1.6 (0.1-13.2)	5.7±14.6 2.3 (0-111.6)	5.2±5.9 2.8 (0.3-27.8)	1.9±2.5 1.1 (0-13.1)	5.2±26.2 1.4 (0.2-255.5)	5.9±22.8 1.9 (0.1-178.3)	<0.05 ^d
Vitamin D (µg/1000 kcal)	1.1	2.7	2.3	1.2	3.4	3.8	
Vitamin E (mg/day) †	8.7±5.1 7.5 (3.6-32.1)	7.9±3.0 7.4 (3.1-18.5)	9.7±4.6 8.4 (3.3-27.3)	6.7±2.4 6.4 (2.4-13.0)	7.1±3.1 6.3 (2.8-22.0)	6.9±2.4 6.5 (2.7-17.3)	<0.05 _{a,b,c}
Vitamin E (mg/100 kcal)	4.2	3.9	4.4	4.1	4.4	4.3	
Niacin (mg/day) †	21.0±11.9 18.4 (7.7-67.1)	21.6±7.7 21.2 (11.0-43.6)	25.1±10.8 23.6 (7.5-70.8)	16.6±8.5 15.6 (5.9-43.4)	17.3±7.3 15.9 (5.7-44.3)	18.9±8.5 17.4 (7.2-45.3)	<0.01 _{b,c}
Niacin (mg/1000 kcal)	9.9	10.5	11.2	10.3	10.9	11.4	
Pantothenic acid (mg/day) †	5.8±2.8 5.3 (2.3-15.8)	5.6±2.0 5.2 (2.7-12.3)	5.9±2.2 5.4 (3.3-15.7)	4.6±1.2 4.4 (3.0-8.1)	4.7±1.3 4.6 (2.2-8.8)	4.9±1.6 4.8 (1.9-11.2)	<0.05 _{a,b,c}
Pantothenic acid (mg/1000 kcal)	2.7	2.7	2.6	2.8	2.9	3.0	
Folates (µg/day) †	311.9±126.7 299.1 (93.5-685.0)	329.8±150.5 315.7 (148.4-1034.5)	393.6±172.9 367.3 (142.9-957.0)	300.4±157.8 270.5 (128.6-1108)	316.6±127.9 308.4 (115.9-642.6)	339.8±164.9 290.2 (90.3-934.6)	>0.05
Folates (µg/1000 kcal)	149.9	164.6	178.7	186.4	201.0	205.9	

†Log-transformed data; *Differences between each level of physical fitness and sex; °Differences within in each sex group. Data are presented as mean±SD or median (min-max). % E: percentage of energy; SFA: saturated fatty acids; MUFA: mono-unsaturated fatty acids; PUFA: polyunsaturated fatty acids; ER, retinol equivalents. ^a Significant differences between low physical fitness groups; ^b Significant differences between medium physical fitness groups; ^c Significant differences between high physical fitness groups; ^d Significant differences in males group. Comparisons between PF groups and sexes with nutrient intake were analysed by One Way Anova or Kruskal-Wallis test, according to the normality of the variables.

Figure 20 presents the percentage of micronutrients below EAR or AI according to sex. More than 85% of males and females showed inadequate intake of potassium, vitamin D and vitamin E. Around 50 % of males and females showed inadequate intake of folate and a total of 82% of females presented inadequate intake of calcium.

Data are presented as percentage (%). †, adequate intake cut-off point.

Figure 20. Percentage of micronutrients below estimated average requirements or adequate intake divided by sex.

Figures 21-22 show the percentage of micronutrients below EAR or AI split by PF groups and sex. There were more participants within low PF group compared to the high PF one which showed inadequate intake for potassium, vitamin D, vitamin E, however, males in the high PF group presented higher inadequate intake compared to low PF group of calcium. Females in the low PF group showed lower inadequate intake than high PF group for calcium, iodine and phosphorous.

Data are presented as percentage (%). †, adequate intake cut-off point.

Figure 21. Percentage of micronutrients below estimated average requirements or adequate intake divided by physical fitness groups in males.

Data are presented as percentage (%). †, adequate intake cut-off point.

Figure 22. Percentage of micronutrients below estimated average requirements or adequate intake divided by physical fitness groups in females.

Additionally, percentage of participants by number of micronutrients intake below EAR or AI is shown in Table 20. A total of 13.0% and 8.3% of the participants presented intakes below EAR or AI for 6 and 11 micronutrients, respectively. Only two participants were above EAR or AI for all micronutrient.

Table 20. Percentage of participants by number of micronutrient (vitamins and minerals) below estimated average requirements or adequate intake.

Number of micronutrient <EAR or AI	Percentage of sample population
0	0.6
1	1.5
2	1.9
3	5.2
4	11.7
5	9.9
6	13.0
7	12.7
8	11.4
9	8.3
10	6.8
11	8.3
12	1.9
13	3.7
14	1.9
15	0.3
16	0.3
17	0.3
18	0
19	0
20	0
21	0

Data are presented as frequency (%).

EAR: estimated average requirements; AI: adequate intake.

Multilevel regression analysis of the PF groups with the usual intake of macro and micronutrients and energy expenditure is presented in Table 21. A positive association was observed for vegetable proteins and high PF group (all $p < 0.05$). At the level of minerals, there were significant and positive associations between participants in the high PF group and phosphorous and selenium (all $p < 0.05$). Furthermore, positive and significant associations were found between high PF group and vitamin B₆, vitamin C, vitamin D, niacin and folates. However, participants in the high PF group presented significant negative associations with thiamine and riboflavin (both $p < 0.05$).

Table 21. Association between physical fitness groups with macro and micronutrients intake and energy expenditure.

Physical fitness groups				
	β	95% CI		<i>p</i> -value
Energy expenditure (METs min/week)	0.000031	-0.000004,	0.000066	0.083
Macronutrients				
Energy (kcal)	0.000143	-0.000105,	0.000392	0.257
Water (mL)	-0.000025	-0.000160,	0.000110	0.718
Proteins (g)†	0.298841	-0.426493,	1.024176	0.058
Vegetable protein (g)†	0.627534	0.021524,	1.233545	0.042
Animal protein (g)†	0.167257	-0.0303647,	0.638160	0.485
Fibre (g)†	0.536173	-0.006146,	1.078492	0.053
Carbohydrates				
Total carbohydrates (g)†	0.078682	-0.662097,	0.819461	0.835
Mono & disaccharides (g)†	0.516633	-0.033544,	1.066810	0.066
Polysaccharides (g)†	-0.026549	-0.453841,	0.400742	0.903
Fats				
Fat (g)	0.003046	-0.001553,	0.007644	0.193
SFA (g)†	0.018181	-0.546022,	0.582384	0.949
MUFA (g)	0.005593	-0.003551,	0.014737	0.230
PUFA (g)†	0.479058	-0.062035,	1.020152	0.082
Cholesterol (mg)†	0.205699	-0.150604,	0.562002	0.257
Alcohol (g)†	0.060786	-0.177779,	0.299351	0.616
Minerals				
Sodium (mg)†	-0.334313	-0.878169,	0.209543	0.227
Potassium (mg)†	1.037949	0.310788,	1.765110	0.005
Magnesium (mg)†	0.419496	-0.201633,	1.040625	0.185
Phosphorous (mg)†	0.803891	0.037866,	1.569916	0.040
Calcium (mg)†	0.156393	-0.412778,	0.725565	0.589
Iron (mg)†	-0.129037	-0.451745,	0.193670	0.432
Copper (mg)†	0.040334	-0.446175,	0.526843	0.871
Selenium (µg)†	0.832301	0.316702,	1.347900	0.002
Iodine (µg)†	-0.078807	-0.616956,	0.459341	0.773
Zinc (mg)†	0.189234	-0.313060,	0.691527	0.459
Vitamins				
Retinol (µg)†	-0.087567	-0.337782,	0.162648	0.492
Carotenes (µg)†	0.255292	0.028529,	0.482054	0.027
Vitamin A (ER)†	0.124278	-0.176778,	0.425335	0.417
Thiamine (mg)†	-0.221961	-0.435925,	-0.007996	0.042
Riboflavin (mg)†	-0.217434	-0.422951,	-0.011917	0.038
Vitamin B ₆ (mg)†	0.442502	0.042278,	0.842726	0.030
Vitamin B ₁₂ (µg)†	-0.054712	-0.309036,	0.199611	0.672
Vitamin C (mg)†	0.344050	0.015266,	0.672834	0.040
Vitamin D (µg)†	0.244887	0.076441,	0.413332	0.005

		β	95% CI	<i>p</i> -value
Vitamin E (mg)†	0.349512	-0.177103,	0.876126	0.193
Niacin (mg)†	0.621547	0.140954,	1.102140	0.011
Pantothenic acid (mg)†	0.465826	-0.187222,	1.118875	0.161
Folates (μg)†	0.621584	0.147431,	1.095736	0.010

Multilevel regression analyses with inclusion of age and sex as covariables. Bonferroni correction resulted in level of significance <0.0013. † Variable was log-transformed to obtain a normal distribution. SFA: saturated fatty acids; MUFA: mono- unsaturated fatty acids; PUFA: polyunsaturated fatty acids.

4.4.5 Discussion

The results from this cross-sectional study showed a remarkable inadequate intake of potassium, vitamin D and vitamin E. It is important to highlight that participants in the low PF group showed inadequate intake for potassium, vitamin D, vitamin E, nevertheless, males in the high PF group presented higher inadequate intake compared to low PF group of calcium. Furthermore, higher macronutrient intakes were observed in the high PF group compared to the other PF groups, but participants in the high PF group had also higher EE, especially in males.

Although the relationship between dietary patterns and PA has been studied (53,58), there is a paucity of data analysing the association of diet and PF in older adults population, concretely in Spain.

In our study, males in the high PF group presented higher EI than in the low PF group (2209 kcal/day vs. 2123 kcal/day, respectively). In females, the mean EI was 1647 kcal/day in the low PF group and 1665 kcal/day in the high PF group. EE was also higher in the high PF group compared to low PF group (males=5205 METs min/week vs. 3863 METs min/week ($p<0.05$); females= 4398 METs min/week vs. 4230 METs min/week, respectively). Camoes et al. found that active males had significantly higher mean intake of energy than sedentary males (2571 kcal/day vs. 2337 kcal/day), respectively (58). Cao et al. in a cross-sectional study observed that compliance with dietary recommendations for macronutrients is significantly associated with higher cardiovascular fitness levels in Japanese male adults (60). Our findings showed that participants in the high PF group showed higher intake of all macronutrients (except total carbohydrates in both sexes and polysaccharides only in females) than in the low PF group. On the contrary, Brodney et al. observed that participants in the high fit category consumed a lower percentage of energy from total fat, saturated fat, monounsaturated fat, and cholesterol and a higher percentage of energy from carbohydrate, and fibre in both sexes (53). Lee et al. found that no significant

differences between groups were obtained for energy and fat intake between PA groups split by METs hours/week (189). Camoes et al. observed a significant lower level of protein consumption (16.9% vs. 17.6% of energy) when comparing active and sedentary males (58). In an earlier study, Rolland et al. observed that active healthy older adults females do not have a better nutritional profile than their inactive peers (296).

The EAR cut-off point method has been accepted as the best method to estimate nutrient intake inadequacy (162). For those nutrients for which an EAR could not be stated, AI was used. In our study, more than 85% of males and females showed inadequate intake of potassium, vitamin D and vitamin E. Román-Viñas et al. observed that vitamin C, folic acid, calcium, selenium, and iodine showed a higher prevalence of inadequate intakes considering EAR cut-off in European elderly (297). Cao et al. found that 25-35% of inadequate intakes for magnesium, calcium and zinc, and may also be relatively high for vitamin A (61.1%) and thiamine (81.0%) in the low PF group (60). Fisberg et al. observed high prevalence of inadequate intake (>50%) of vitamins D, E and A, calcium, magnesium and pyridoxine (112). It is important to highlight in our study that participants in the low PF group showed inadequate intake for potassium, vitamin D, vitamin E, nevertheless, males in the high PF group presented higher inadequate intake compared to low PF group of calcium. In addition, around 8% of the participants presented inadequate intakes in 11 micronutrients in our study.

Likewise, participants from the high PF groups showed greater mean intake of potassium, magnesium, phosphorous, selenium and vitamins (B₃, B₅, B₆, B₉, C, D, E) and retinol (only females) than low PF groups. Cao et al. showed that participants who had a poor overall micronutrient intake status have a significantly higher risk of being unfit compared to male with a good micronutrient intake status (60). In this sense, Brodney et al. observed that high fit category consumed higher percentage of energy from calcium, folate, vitamin B₆, vitamin B₁₂ (only female), vitamin A, vitamin C, and vitamin E than the low and moderate fit in both sexes (53). Because micronutrients are essential for humans health (185), it is important to take into account the inadequate intake of vitamins and minerals in order to prevent deficiencies and considering the influence in the different biological pathways of micronutrient over PF. Long period of inadequate intake of one or some micronutrients may display clinical manifestations of deficiency (112).

The findings of our study also showed that a positive and significant association was observed for vegetable protein and high PF group. There were significant and positive associations between high PF group with phosphorous and selenium. Furthermore, positive and significant associations were found between high PF group and vitamin B₆, vitamin C, vitamin D, niacin, and folates. However, high PF group presented negative associations with thiamine and riboflavin. Camoes et al. observed a significant and positive association between PA and intake of vitamin C. Additionally, leisure-time activity was positively associated with intakes of vitamin E, folate, calcium and magnesium in females (58). Other authors who studied the association between PF and diet quality observed that diet-quality score was positively associated with cardiorespiratory fitness (322) and more physical exercise was associated with improvement of the Mediterranean Diet Adherence (266).

As shown above, data on micro and macronutrient intake, obtained by different authors, were discrepant. This could be mainly due to different approaches when assessing the quantity of a particular micro and macronutrient in a particular kind of food, or differences in diet questionnaires used. A considerable heterogeneity exists in dietary patterns and nutritional status in old population (141). In addition, dietary intake tends to decline over time (303). Overall, diet and PF modifications seem to be strong promoters of healthy ageing (141).

Limitations and strength

This study has several limitations. Firstly, one limitation of the study was the use of 24h dietary recalls. The 24h dietary recall method is one of the most widely used tools and validated methods (310); however, the 24h dietary recall method does not allow quantifying proportions of non-consumers for particular for items, especially for infrequently consumed foods. Secondly, this study has a cross-sectional design, thus preventing the determination of cause-effect relationships. Nevertheless, in this study, it seems reasonable to think that macro and micronutrient status can be associated to PF, whereas the mechanisms by which PF could determine higher or lower intake status is not so clear.

Despite of the aforementioned, this study also has several strengths which included the use objective, precise and validated method of PF tests for assessing health markers. Furthermore, the use of clustering of activities produces an alternative approach to

summarizing PF participation. Another strength is the sampling procedure and the strict standardization of the field work among the cities involved in the study avoiding to a great extent the kind of confounding bias.

4.4.6 Conclusions

In conclusion, higher macronutrient intake were observed in the high PF group compared to the other PF groups, but participants in the high PF group had also higher EE, especially in males. Participants in the high PF group showed a better micronutrient intake profile than the others PF groups. PF level seems to be associated with nutrient intake. Additionally, there are a considerable number of participants who presented inadequate intake of micronutrients below the EAR. There is a requirement to develop nutritional programs and fitness among older adults and to consider supplementation for micronutrients at risk.

Annex 1. Number of participants in each PF group considering non-underreporters and all sample.

Non-underreporters (n=324)						
Male				Female		
	Low	Medium	High	low	Medium	High
n	30	59	41	37	95	62
All sample (n=428)						
Male				Female		
	Low	Medium	High	low	Medium	High
n	43	86	55	53	121	70

n, no.of participants.

Annex 2. Energy expenditure, macronutrients and micronutrients intake split by physical fitness groups in underreporters participants.

Underreporter (n=104)			
	Low fitness (n=34)	Medium fitness (n=48)	High fitness (n=22)
	Mean±SD	Mean±SD	Mean±SD
	Median (min-max)	Median (min-max)	Median (min-max)
Energy expenditure	4144±1826	4422±2190	5708±3165
(METs min/week)	3692 (1208-8135)	4206 (1350-10416)	4788 (1024-12359)
Macronutrients			
Energy (Kcal/day)	1163±283.6	1292±254.4	1291±247.0
	1120 (540.7-1636)	1301 (722.2-1865)	1342 (884.4-1725)
Water (ml/day)	2170±650.4	1914±650.3	2232±777.6
	2153 (1123-3789)	1800 (677.2-3803)	1903 (1191-3655)
Proteins (g/day)†	51.8±15.8	58.1±14.3	58.8±17.5
	51.3 (27.3-97.5)	57.7 (21.1-100.4)	54.8 (36.7-93.3)
Proteins (% E)	17.7	18.1	18.1
Vegetable protein	14.0±3.8	15.7±4.4	16.2±5.7
(g/day)†	14.0 (5.3-20.3)	16.7 (5.7-25.1)	15.5 (6.8-29.3)
Animal protein (g/day)†	35.5±15.4	40.5±14.7	40.6±14.3
	29.3 (9.9-79.6)	39.8 (8.0-76.5)	33.2 (24.0-70.1)
Fibre (g/day)†	14.2±4.9	14.3±5.0	17.5±5.5
	12.9 (7.3-27.4)	13.7 (6.0-26.6)	16.8 (7.2-29.0)
Fibre (g/1000 kcal)	12.9	11.4	13.8
Carbohydrates			
Carbohydrates (g/day)†	135.9±38.7	143.4±38.2	148.2±34.8
	138.4 (29.2-205.4)	144.4 (43.4-211.8)	141.6 (86.6-209.9)
Carbohydrates (% E)	47.1	44.6	45.9
Mono & disaccharides	69.6±27.0	71.3±28.0	89.0±18.6
(g/day)†	65.8 (28.1-147.1)	66.8 (16.5-145.7)	82.8 (62.0-130.8)
Polysaccharides (g/day)†	53.6±24.9	64.1±31.3	54.4±25.9
	47.2 (1.1-100.0)	65.2 (2.1-135.3)	57.7 (7.1-97.9)
Fats			
Fat (g/day)	41.6±14.7	49.0±16.8 (15.4-88.1)	45.7±12.3
	41.0 (9.0-79.9)		46.0 (17.8-69.7)
Fat (% E)	32.9	34.6	31.4
SFA (g/day) †	11.8±4.7	13.9±5.7	13.1±4.9
	12.1 (2.9-23.4)	13.3 (4.7-26.7)	11.6 (7.5-25.3)
SFA (% E)	8.9	9.5	9.0
MUFA (g/day)	18.3±7.6	23.2±9.4	20.6±6.3
	36.1 (2.3-38.4)	21.2 (4.1-45.9)	20.3 (5.6-31.0)
MUFA (% E)	14.0	15.9	14.5
PUFA (g/day)† ^b	5.4±2.4	6.0±2.1	6.1±2.3
	4.6 (2.1-12.3)	6.1 (2.0-11.2)	5.7 (2.0-12.3)
PUFA (% E)	4.2	4.2	4.3

	Low fitness (n=34)	Medium fitness (n=48)	High fitness (n=22)
	Mean±SD	Mean±SD	Mean±SD
	Median (min-max)	Median (min-max)	Median (min-max)
Cholesterol (mg/day)†	234.2±141.8	232.2±140.0	222.7±132.1
Cholesterol (mg/1000 kcal)	222.9 (41.3-507.2) 198.5	183.3 (18.2-590.2) 178.6	171.7 (85.0-527.9) 171.7
Alcohol (g/day) †	5.9±8.2 0 (0-28.8)	6.9±9.2 4.8 (0-44.0)	7.6±10.0 1.8 (0-33.6)
Alcohol (% E)	3.5	3.2	4.8
Minerals			
Sodium (mg/1000 kcal)†	1125	1104	931.3
Potassium (mg/1000 kcal)†	1907	2000	2250
Magnesium (mg/1000 kcal)†	188.3	175.8	217.2
Phosphorous (mg/1000 kcal)†	718.3	719.6	752.6
Calcium (mg/1000 kcal)†	501.8	499.8	533.8
Iron (mg/1000 kcal)†	12.7	15.8	8.7
Copper (mg/1000 kcal)†	1.4	1.3	1.5
Selenium (mg/1000 kcal)†	63.4	63.0	59.8
Iodine (µg /1000 kcal)†	101.2	94.8	96.9
Zinc (mg/1000 kcal)†	5.8	8.7	5.5
Vitamins			
Retinol (µg /1000 kcal)†	278.2	329.5	138.3
Carotenes (µg /1000 kcal)†	2979	2969	2821
Vitamin A (ER/1000 kcal)†	777.3	838.5	618.3
Thiamine (mg/1000 kcal)†	3.3	1.8	1.2
Riboflavin (mg/1000 kcal)†	4.6	2.5	2.1
Vitamin B ₆ (mg/1000 kcal)†	1.1	1.1	1.6
Vitamin B ₁₂ (µg /1000 kcal)†	5.2	4.6	5.8

	Low fitness (n=34)	Medium fitness (n=48)	High fitness (n=22)
	Mean±SD	Mean±SD	Mean±SD
	Median (min-max)	Median (min-max)	Median (min-max)
Vitamin C (mg/1000 kcal)†	98.9	102.1	133.6
Vitamin D (µg/1000 kcal)†	82.2	8.9	1.5
Vitamin E (mg/100 kcal)†	4.6	4.3	5.0
Niacin (mg/1000 kcal)†	10.3	12.4	13.5
Pantothenic acid (mg/1000 kcal)†	3.4	3.6	3.7
Folates (µg/1000 kcal)†	255.8	216.4	240.9

†Log-transformed data. Data are presented as mean±SD or median (min-max) for macronutrients and alcohol. n, no.of participants. Data are presented as units/1000 kcal for micronutrients. % E: percentage of energy; SFA: saturated fatty acids; MUFA: mono-unsaturated fatty acids; PUFA: polyunsaturated fatty acids; ER, retinol equivalents.

4.5 ARTICLE 5: DIFFERENT ASSOCIATIONS BETWEEN PHYSICAL FITNESS, PHYSICAL ACTIVITY AND SEDENTARISM WITH OXIDATIVE STRESS PARAMETERS DEPENDING ON SEX AMONG SPANISH OLDER ADULTS

Aparicio-Ugarriza R, Pedrero-Chamizo R, Palacios G, Bibiloni MM, Sureda A, Tur JA, González-Gross M. *Exp Gerontol* (submitted). (JCR: 3.350).

4.5.1 Abstract

The aim of this study was to analyse the association between physical fitness (PF), physical activity (PA), sedentary behaviour, antioxidant nutrients and parameters linked with unbalanced cell oxidative equilibrium among Spanish old adults. **Methods:** A total of 404 participants aged over 55 yr (57% females) from Madrid and Mallorca (Spain) were recruited for the study. Malondialdehyde (MDA) was analysed from all participants. A maximum of 164 participants were studied for superoxide dismutase (SOD), catalase (CAT), myeloperoxidase (MPO), glutathione peroxidase and glutathione reductase from Mallorcan participants. PF was assessed by means of a validated battery and PA and sedentary behaviour through a validated short tool. Also, antioxidants intake was measured by of 24h dietary recalls and nutrient intake was calculated using ALIMENTA software. Alcohol intake was evaluated by means of a hydration questionnaire. **Results:** In males, MPO was a strongly relationship with increased vitamin E ($\beta=0.44$, $p<0.001$) and decreased less age ($\beta=-0.34$, $p<0.01$), explaining 28% of the variance. SOD in lymphocytes was strong associated with increased PF level ($\beta=0.39$, $p<0.001$). In females, MDA was negatively associated with PF level ($\beta= -0.16$, $p=0.02$) and CAT in plasma showed a negative relationship with vitamin E ($\beta= -0.24$, $p=0.03$). Also, SOD lymphocytes was positive linked with alcohol and SOD plasma with age (both $\beta=0.26$, $p=0.03$). **Conclusion:** Different behaviours were found between sexes and oxidative stress parameters, thus, our data indicate that some oxidative stress parameters are related to lifestyle behaviour and sex should be considered in further studies.

Key words: oxidative stress; lipid peroxidation; physical fitness; sedentary lifestyle; aging.

4.5.2 Introduction

Aging is associated with an oxidative imbalance: antioxidant enzymes diminish their activity against free radicals which are created by the mitochondrial respiratory chain (301). Oxidative stress is a major determinant of lifespan and provides to a growing reduction in cell function and accelerating aging timing (79), and it is declined as the result of the imbalance between pro-oxidant and antioxidant substances. The balance between free-radical generation and antioxidant activity is critical to the pathogenesis of oxidative stress-related disorders (78).

Physical activity (PA) increases generation of Reactive Oxygen Species (ROS), which have a high oxidising capacity (116). However, ROS are important signalling molecules involved in crucial biological process at suitable levels such as muscle concentration, which are essential mechanisms to avoid cell damage after PA. Likewise, an excess of ROS can produce oxidative damage (376).

The activity of antioxidant enzymes in response to the accumulation of ROS is equivocal, and both increased and decreased activities have been studied during aging processes (8). Nevertheless, if defence mechanism fails or presents abnormal functioning, oxidative stress occurs (8). Increased ROS react with polyunsaturated fatty acids to induce malondialdehyde (MDA) which is a marker of lipid peroxidation and its related to Alzheimer and Parkinson disease, cancer, among others (177,276).

Genetic factors, environment, diet, sedentary behaviour and physical inactivity could induce abnormal increase in free radical production and/or decrease in antioxidant defence. Thus, all these variables may tip the overall balance between antioxidant and prooxidant toward increased levels of oxidant stress (204). Additionally, physical fitness (PF) could play an important role in the oxidative stress balance/imbalance. It is well documented that PF is considered as health marker (42,256,269) and low levels of PF has been related to increase risk of several diseases, disabilities and higher risk of mortality (98,128). Regular exercise, moderate intensity exercise and long term (at least 6 months) are vital for older population to prevent the generation of free radicals (195). Nevertheless, available data concerning influence of PA and PF on the level of antioxidant barrier is controversial (124).

Therefore, it still necessary to study the relationship between PF and oxidative stress parameters, especially in older adults due to the importance of PF related to health and oxidative stress. The aim of this study was to examine the relationship between PF, PA,

sedentary behaviour, antioxidant nutrients and parameters related to unbalanced cell oxidative equilibrium among Spanish aged over 55 yr.

4.5.3 Materials and Methods

Study design, sample and ethics

The PHYSMED project is a multi-centre cross-sectional study aimed at identifying cardiovascular risk factors in sedentary and active old population. Data were collected from April 2013 to May 2014 and field work took place in 2 Spanish cities: Madrid and Mallorca. Volunteers were recruited through health centres, sport federations, sport facilities and municipal clubs located at Madrid and Mallorca through snowball system. The study population consisted of 433 adults (57% females), aged 55-88 yr. For the purpose of this study, participants who provided data on oxidative stress parameters were included in the analysis, resulting in 404 participants for MDA from both cities and a maximum of 164 participants for superoxide dismutase (SOD), catalase (CAT), myeloperoxidase (MPO), glutathione peroxidase (GPx) and glutathione reductase (GRd) from Mallorcan participants. Individual exclusion criteria were not aged under 55 yr, being institutionalized, suffering from a physical or mental illness which would have limited their participation in the physical fitness tests or their ability to respond to the questionnaires or drug intake for clinical research.

The study was conducted following the ethical guidelines of the Declaration of Helsinki and the protocol was approved by the Ethical Committee of the Technical University of Madrid. Written informed consent was obtained from all participants.

Oxidative stress parameters

Fasted (12h) blood samples were collected from each participant. The extraction was performed by standard venepuncture on vacuum Vacutainer® tubes with separation gel. The tubes were immediately placed on ice and after coagulum formation the sample was centrifuged at 3,000 rpm for 10 min. Oxidative stress parameters were centralized at the University of Balearic Islands and thus, samples were dry-ice shipped from Madrid to Mallorca. Because enzymatic activity measures could only be performed on fresh blood, samples dry-ice shipped from Madrid to Mallorca consisted of only erythrocytes hemolysates and leucocytes concentrates for enzyme concentrations. Thus, enzyme activity data came only from Mallorca samples.

SOD activity was determined in plasma by an adaptation of the method of McCord and Fridovich (221), and measured with a Shimadzu UV-2100 spectrophotometer at 37°C. CAT activity was measured in neutrophils by the spectrophotometric method of Aebi (3) based on the decomposition of H₂O₂. MPO activity of neutrophils was measured by guaiacol oxidation (61). The reaction mixture contained sodium phosphate buffer, pH 7.0 and 13.5 mmol/L of guaiacol. The reaction was initiated by adding 300 mol/L of H₂O₂, and changes at 470 nm were monitored. GPx activity was measured using an adaptation of the spectrophotometric method of Flohé and Gunzler (114). This activity was determined both with H₂O₂ and cumene hydroperoxide as substrates and with GRd and NADPH as enzyme indicators. GRd activity was measured in neutrophils by a modification of the Goldberg and Spooner spectrophotometric method which required oxidized glutathione as the substrate (127). The activities of CAT, MPO, GPx and GRd in neutrophils were measured with a Shimadzu UV-2100 spectrophotometer at 37°C. MDA as a marker of lipid peroxidation was analysed in neutrophils using a colorimetric assay kit (Calbiochem). Briefly, samples and standards were placed in Eppendorfs containing N-methyl-2-phenylindole (10.3 mmol/L) in acetonitrile: methanol (3:1, v/v). HCl (12 mol/L) was added and samples were incubated for 1h at 45°C. Absorbance was measured at 586 nm.

Physical fitness

Prior to assess PF tests, blood pressure was measure using a digital monitor (Omron Healthcare Co., Ltd. Kyoto, Japan). A battery of a multi-component of PF tests validated in the elderly population (290) and validated reference ranges for Spanish older adults proposed by Pedrero-Chamizo et al. were completed by each participant (269). Lower body strength was measured by the chair stand test (290), agility/dynamic balance was performed by the 8-foot up-and-go test (290), aerobic endurance was assessed by the 6-min walk test (290) and handgrip strength was measured with a handgrip dynamometer (361) (Takei TKK 5401, Tokyo, Japan, range 5-100 kg, precision 0.1 kg). The handgrip strength was assessed for both hands in a standing position. All tests were performed twice, except the 6-min walk test and the chair stand test, and the best score was retained.

The results of each PF tests were stratified by sex and age groups (distributed by five years periods without truncating the last group) following the criteria established by

Pedrero-Chamizo et al. (269). Also, the result of each PF test was divided into quartiles considering the variables above mentioned (sex and age groups). The score for each test ranged from 0 (worst) to 3 (best) points. Thus, the maximum score was 12 points. Scores of PF tests were added together to create a cluster and participants were divided into three groups: low, medium and high.

Physical activity and sedentarism

Our population was classified into 4 groups: inactive and low sedentary (ILS), inactive and high sedentary (IHS), active and high sedentary (AHS) and active and low sedentary (ALS) taking into consideration the classification proposed by Aparicio-Ugarriza et al. (22).

Anthropometric measurements

Height (m) was assessed to the nearest millimetre using a mobile stadiometer (SECA 213, Germany), with the participant's head in the Frankfurt plane. Body weight (kg) was measured using bioimpedance analysis (TANITA Corp, BC-418MA, Tokyo, Japan) in standardized conditions. Participants were barefoot and in light clothing during anthropometric measurements. Body mass index (BMI) was calculated as the weight (kg) divided by the square of the height (m^2). Also, BMI was divided into three categories: normal weight ($<24.9 \text{ kg}/m^2$), overweight ($25.0\text{-}29.9 \text{ kg}/m^2$) and obese ($>30.0 \text{ kg}/m^2$).

Furthermore, waist circumference was measured as the smallest horizontal girth between the costal margins and the iliac crests at minimal respiration using a flexible, non-extensible plastic tape with 0.1 cm precision (Rosscraft), according to the methods of the ISAK society (210). Waist to height ratio (WHtR) was defined as waist circumference (cm) divided by height (cm). Additionally, a WHtR cut-off of 0.580 was considered following the criteria proposed by Bohr et al. (46).

Smoking habits, nutrient intake and alcohol

The ExQ included questions about smoking habits and participants were grouped as follows: smoker and non-smoker.

Regarding nutrient intake, dietary intake was obtained by means of 24h dietary recalls by well-trained dieticians. The 24h dietary recalls were collected on two non-

consecutive days within a period of 2-3 weeks. Selenium, vitamin A, vitamin C and vitamin E were calculating using software (ALIMENTA; NUCOX, Palma, Spain) based on Spanish (218,255) and European (106) food composition tables, and complemented with food composition data available for Mallorcan food items (291). To better show the effects, micronutrient intake was categorized below estimated average requirements (EAR) (162). Additionally, fruit intake per day was recorded and encoded as follows: (i) one a day, (ii) 2-3 fruits/day and (iii) >4 fruits/day.

Alcohol intake (mL/day) was measured by means of a hydration questionnaire (213). Beer, wine and distilled drinks were added and 3 categories were established as follows: (i) 0 mL/day, (ii) 0.1-149.9 mL/day and (iii) 150.0 mL/day.

Statistical analysis

Statistics analyses were performed using statistical software SPSS (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp). Descriptive characteristics were summarized by calculating median and range unless otherwise stated. All variables of PF tests were checked for normality of distribution by Kolmogorov-Smirnov test and all variables showed normal distribution. All oxidative parameters, micronutrient intake and alcohol showed a non-normal distribution and log-transformation was done.

Comparisons between oxidative stress parameters and PF, PA and sedentary behaviour, lifestyles variables, body composition and micronutrient intake were analysed by Jonckheere trend test. Likewise, age was categorized to the best understanding as follows: (i) <64.9 yr, (ii) 65.0-74.9 yr, (iii) >75 yr. Variables that showed significant association with one or more enzymes activities were included in the linear regression model. Values of $p < 0.05$ were considered statistically significant.

4.5.4 Results

Table 22 summarizes the characteristics of the study population.

Table 22. Characteristics of participants split by sex.

Characteristics	Male n (%)	Female n (%)
Age (yr)		
<64.9	45 (25.7)	25 (10.9)
65.0-74.9	80 (45.7)	126 (55.0)
>75.0	50 (28.6)	78 (34.1)
Physical fitness		
Low	50 (28.6)	72 (31.4)
Medium	71 (40.6)	92 (40.2)
High	54 (30.9)	65 (28.4)
Physical activity and sedentary behaviour		
Inactive and high sedentarism	82 (46.9)	93 (40.6)
Inactive and low sedentarism	30 (17.1)	67 (29.3)
Active and high sedentarism	37 (21.1)	33 (14.4)
Active and low sedentarism	26 (14.9)	36 (15.7)
Smoking status		
Non-smoker	160 (91.4)	210 (92.1)
BMI (kg/m²)		
Normal weight	39 (22.3)	83 (36.2)
Overweight	101 (57.7)	100 (43.7)
Obese	35 (20.0)	46 (20.1)
WHtR		
Low WHtR (<0.58)	101 (57.7)	171 (74.7)
Alcohol intake (mL/day)		
0	152 (86.9)	226 (98.7)
0.1-149.9	5 (2.9)	1 (0.4)
>150.0	18 (10.3)	2 (0.9)
Fruit intake		
Once a day	39 (22.5)	32 (14.1)
2-3/day	91 (52.0)	133 (58.6)
>4/day	43 (24.6)	62 (27.3)
Selenium intake (µg)		
<EAR	7 (4.0)	17 (7.5)
Vitamin A intake (ER)		
<EAR	59 (34.1)	64 (28.2)
Vitamin E intake (mg)		
<EAR	155 (89.6)	218 (96.0)
Vitamin C intake (mg)		
<EAR	39 (22.5)	20 (8.8)

BMI, body mass index; WHtR, waist to height ratio; EAR, estimated average requirements; ER, retinol equivalents.

Tables 23-24 show the comparisons between several oxidative stress parameters and PF, PA and sedentary behaviour, body composition, fruit intake, vitamins and minerals intake and alcohol in males and females, respectively.

MDA showed significant association with age (for both sexes), vitamin E (males), PF and vitamin E only in females ($p<0.05$). A significant relationship was observed between CAT and age, BMI and selenium in males ($p<0.05$), whereas CAT Lmf was significant linked with PF and fruits in females ($p<0.05$). On the other hand, in males, age, PF, alcohol and selenium intake was significantly associated with CAT Plm ($p<0.05$). In females, CAT Plm only showed significant relationship with PA and sedentary groups and vitamin C ($p<0.05$). Likewise, SOD Lmf was significantly linked with age and PF in males. Additionally, SOD in plasma showed a significant relationship with PF and selenium intake (in males) and age (females) ($p<0.05$). GPx did not show significant association in both sexes but a significant association was observed between GRd and PF only in males ($p<0.05$). In males, a significant relationship was found between MPO and age, vitamin A and E. In females, MPO showed a significant association with PF, age and vitamin A.

Table 23. Associations between oxidative stress parameters and health markers, age and dietary factors in males.

Variables	n	Co MDA (uM)	n	CAT Lmf (K/10 ⁹ Cells)	n	SOD Lmf (pKat/10 ⁹ Cells)	n	GPX Lmf (nkat/10 ⁹ Cells)	n	GRd Lmf (nkat/10 ⁹ Cells)	n	MPO Lmf (nkat/mL)	n	CAT Plm (k/L)	n	SOD Plm (pkat/L)
Median (range)	175	1.4 (0.1- 21.1)	81	7.1 (1.3- 510.1)	72	53.5 (2.0- 377.9)	76	41.3 (8.0- 249.4)	67	185.0 (28.4- 2407)	79	20853 (0- 912138)	80	17.4 (3.9- 235.5)	78	605.6 (112.2- 1787)
Age (yr)																
<64.9	80	1.3 (0.1- 16.2)	42	14.0 (1.4- 307.8)	39	61.1 (6.7- 377.9)	41	42.1 (10.3- 249.4)	37	243.8 (28.4- 2407)	42	325559 (6761- 812138)	42	25.1 (4.8- 235.5)	41	534.0 (135.- 1175)
65.0-74.9	79	1.4 (0.4- 21.1)	35	4.7 (1.3- 167.2)	29	51.0 (2.0- 254.8)	33	36.0 (8.0- 170.4)	26	130.6 (41.4- 1174)	33	16578 (0- 468707)	34	12.8 (3.9- 110.8)	33	619.1 (112.2- 1787)
>75.0	16	1.4 (0.7- 1.9)	4	11.4 (3.4- 510.1)	4	47.2 (24.7- 75.7)	2	44.5 (18.5- 70.4)	4	111.0 (71.0- 470.4)	4	14075 (8387- 34298)	4	96.4 (18.1- 173.9)	4	506.9 (387.9- 698.7)
<i>p</i> -trend		0.04		0.03		0.08		0.66		0.45		0.00		0.09		0.86
Physical fitness																
Low	50	1.4 (0.3- 21.1)	31	5.6 (1.3-510.1)	28	36.8 (2.0- 118.4)	32	39.1 (11.2-218.7)	27	132.2 (28.4-673.7)	32	19999 (0-468707)	33	15.1 (4.8- 173.9)	32	568.1 (112.2- 1431)
Medium	71	1.4 (0.1- 19.2)	34	19.7 (1.4-307.8)	30	58.7 (17.1-377.9)	29	47.0 (10.3-174.8)	28	309.4 (33.2-1174)	31	19713 (6332- 515804)	31	17.5 (3.9- 235.5)	31	580.3 (135.3- 1011)
High	54	1.4 (0.2- 3.5)	16	5.0 (1.4-74.9)	14	85.1 (18.2-371.7)	15	37.8 (8.0-249.4)	12	131.1 (42.3-2407)	16	121857 (0-812138)	16	27.5 (7.3- 179.2)	15	625.0 (456.2- 1787)
<i>p</i> -trend		0.52		0.52		0.03^{ns}		0.60		0.09		0.26		0.06		0.04

Variables	n	Co MDA (uM)	n	CAT Lmf (K/10 ⁹ Cells)	n	SOD Lmf (pKat/10 ⁹ Cells)	n	GPX Lmf (nkat/10 ⁹ Cells)	n	GRd Lmf (nkat/10 ⁹ Cells)	n	MPO Lmf (nkat/mL)	n	CAT Plm (k/L)	n	SOD Plm (pkat/L)
Physical activity and sedentary behaviour groups																
Inactive and high sedentarism	82	1.4 (0.4-21.1)	43	10.5 (1.3-510.1)	37	51.4 (2.0-377.9)	42	41.3 (8.0-218.7)	35	200.0 (28.4-2407)	42	20619 (0-515805)	44	13.8 (3.9-173.9)	41	610.7 (135.3-1787)
Inactive and low sedentarism	30	1.3 (0.1-16.2)	14	4.7 (3.0-90.3)	14	46.9 (18.2-254.8)	14	47.0 (13.4-170.4)	12	146.5 (42.3-1174)	14	15629 (4249-382933)	14	16.2 (5.9-72.2)	14	535.5 (112.2-1431)
Active and high sedentarism	37	1.4 (0.2-19.2)	17	5.6 (1.5-307.8)	14	52.2 (12.0-272.4)	13	36.0 (10.3-249.4)	16	136.4 (33.2-941.8)	16	18164 (7500-812138)	17	24.8 (5.4-235.4)	16	642.1 (398.0-916.0)
Active and low sedentarism	26	1.4 (0.4-2.2)	7	5.4 (4.7-74.8)	7	70.3 (33.8-371.7)	7	47.0 (30.9-174.8)	4	245.0 (71.0-1166)	7	34298 (18788-476300)	5	86.2 (18.1-179.2)	7	516.2 (158.8-947.1)
<i>p</i> -trend		0.56		0.88		0.20		0.50		0.96		0.75		0.06		0.93
Smoking status																
Smoker	15	1.4 (0.5-16.2)	7	28.6 (2.5-69.3)	5	38.2 (2.0-48.7)	7	39.8 (14.5-218.7)	6	346.4 (45.8-527.9)	6	33486 (0-468707)	7	9.6 (5.2-132.4)	6	647.7 (201.2-787.5)
Non-smoker	160	1.4 (0.1-21.1)	74	6.0 (1.3-510.1)	67	56.3 (6.7-377.9)	69	41.6 (8.0-249.4)	61	136.9 (28.4-2407)	73	20853 (0-812138)	73	18.1 (3.9-235.5)	72	605.6 (112.2-1787)
<i>p</i> -value		0.76		0.47		0.12		0.69		0.66		0.43		0.19		0.92
BMI (kg/m²)																
Normal weight	39	1.4 (0.3-3.5)	16	4.5 (2.5-74.8)	15	70.3 (23.5-371.7)	15	44.9 (15.2-218.7)	14	101.7 (45.8-1167)	16	22372 (4249-476300)	15	30.8 (5.4-235.5)	16	674.1 (158.8-1431)
Overweight	101	1.4 (0.2-19.2)	44	6.0 (1.3-510.1)	40	46.9 (2.0-272.4)	42	37.1 (8.0-249.4)	37	191.1 (33.2-2407)	44	20348 (0-812138)	44	16.0 (4.8-173.9)	43	537.0 (135.-1787)
Obese	35	1.5 (0.1-21.1)	21	19.6 (1.4-307.8)	17	56.3 (6.7-377.9)	19	47.6 (10.5-163.9)	16	365.7 (28.4-941.8)	19	24238 (6332-515805)	21	25.4 (3.9-160.0)	19	682.8 (112.2-1175)
<i>p</i> -trend		0.60		0.06 ^{gh}		0.67		0.57		0.23		0.96		0.49		0.75

Variables	n	Co MDA (uM)	n	CAT Lmf (K/10 ⁹ Cells)	n	SOD Lmf (pKat/10 ⁹ Cells)	n	GPX Lmf (nkat/10 ⁹ Cells)	n	GRd Lmf (nkat/10 ⁹ Cells)	n	MPO Plm (nkat/mL)	n	CAT Plm (k/L)	n	SOD Plm (pkat/L)
WHtR																
Low WHtR (<0.58)	101	1.4 (0.2- 19.2)	46	5.2 (1.3- 510.1)	42	57.0 (15.3- 371.7)	45	38.9 (8.0- 249.4)	41	228.5 (33.2- 2407)	46	22012 (0- 812138)	45	19.3 (4.8- 235.5)	45	580.3 (135.3- 1787)
High WHtR (≥ 0.58)	74	1.4 (0.1- 21.1)	35	9.1 (1.4- 307.8)	30	52.4 (2.0- 377.9)	31	42.4 (10.5- 163.9)	26	134.0 (28.4- 941.8)	33	20853 (0- 515805)	35	16.8 (3.9- 160.0)	33	639.9 (112.2- 1175)
<i>p</i> -value		0.18		0.23		0.43		0.86		0.27		0.82		0.76		0.86
Alcohol (ml/day)																
0	152	1.4 (0.1- 19.2)	70	6.0 (1.3- 510.1)	62	53.5 (2.0- 371.7)	68	41.3 (8.0- 249.4)	56	184.2 (28.4- 2406)	69	21640 (0- 812138)	69	16.8 (3.9- 179.2)	68	598.5 (119.2- 1787)
0.1-149.9	5	1.1 (0.5- 2.0)	4	43.1 (2.1- 107.6)	4	96.2 (18.8- 377.9)	3	64.2 (10.3- 99.2)	4	425.7 (33.2- 897.4)	4	154896 (7500- 515805)	4	86.7 (10.2- 235.5)	4	804.3 (397.9- 946.4)
>150.0	18	1.5 (0.5- 21.1)	7	7.1 (3.4- 307.8)	6	44.7 (24.7- 254.7)	5	37.2 (18.5170.4)	7	135.8 (68.2- 597.7)	6	13175 (8387- 363968)	7	38.3 (10.0- 110.8)	6	628.4 (461.0- 770.6)
<i>p</i> -trend		0.42		0.24		0.56		0.89		0.93		0.53		0.09		0.52
Fruit																
Once a day	39	1.5 (0.3- 16.2)	20	8.8 (1.4- 97.1)	18	73.2 (22.3- 371.7)	20	36.8 (14.5- 158.1)	16	308.9 (50.2- 2407)	20	26065 (0- 812138)	20	16.0 (4.8- 235.5)	19	715.8 (279.9- 1787)
2-3/day	91	1.4 (0.2- 10.8)	39	5.8 (1.3- 307.8)	33	44.6 (12.3- 377.9)	38	43.5 (8.0- 218.7)	34	128.7 (41.4- 1166)	37	18788 (1632- 515805)	38	16.8 (3.9- 171.8)	37	579.7 (135.3- 1175)
>4/day	43	1.4 (0.2- 10.8)	22	12.4 (1.5- 510.1)	21	60.5 (2.0- 254.8)	18	39.2 (10.3- 249.4)	17	135.7 (28.4- 1174)	22	27076 (7500- 468707)	22	22.8 (5.2- 173.9)	22	604.8 (112.2- 1431)
<i>p</i> -trend		0.83		0.77		0.45		0.96		0.16		0.78		0.70		0.13

Variables	n	Co MDA (uM)	n	CAT Lmf (K/10 ⁹ Cells)	n	SOD Lmf (pKat/10 ⁹ Cells)	n	GPX Lmf (nkat/10 ⁹ Cells)	n	GRd Lmf (nkat/10 ⁹ Cells)	n	MPO Plm (nkat/mL)	n	CAT Plm (k/L)	n	SOD Plm (pkat/L)
Selenium intake (µg)																
<EAR	7	1.3 (0.6- 2.3)	4	3.6 (1.4- 5.5)	4	36.0 (29.3- 96.2)	4	55.6 (14.8- 70.4)	3	71.0 (45.8- 228.5)	4	18169 (9403- 34298)	4	11.1 (4.8- 18.1)	4	468.7 (112.2- 531.0)
>EAR	166	1.4 (0.1- 21.1)	77	8.5 (1.3- 510.1)	68	53.5 (2.0- 377.9)	72	40.4 (8.0- 249.4)	64	188.1 (28.4- 2406)	75	21640 (0- 812138)	76	18.4 (3.9- 235.5)	74	612.7 (135.3- 1787)
<i>p</i> -value		0.64		0.07		0.49		0.83		0.17		0.44		0.09		0.06
Vitamin A intake (ER)																
<EAR	59	1.4 (0.4- 10.8)	20	5.4 (1.3- 510.1)	19	38.1 (15.3- 371.7)	17	29.5 (8.0- 96.3)	18	115.5 (41.4- 941.8)	19	16577 (1632- 418110)	19	13.5 (4.8- 179.2)	19	619.1 (319.7- 1012)
>EAR	114	1.4 (0.1- 21.1)	61	8.5 (1.4- 307.8)	53	56.3 (2.0- 377.9)	59	45.3 (10.3- 249.4)	49	243.8 (28.4- 2406)	60	28793 (- 812138)	61	19.4 (3.9- 235.5)	59	584.5 (112.2- 1787)
<i>p</i> -value		0.80		0.33		0.23		0.03		0.06		0.01		0.55		0.43
Vitamin E intake (mg)																
<EAR	155	1.4 (0.1- 21.1)	71	8.5 (1.3- 510.1)	62	51.2 (6.7- 371.7)	66	41.3 (8.0- 218.7)	60	188.1 (28.4- 1174)	69	19688 (0- 812138)	70	17.0 (3.9- 235.5)	69	591.7 (112.2- 787)
>EAR	18	1.6 (0.9-3.5)	10	5.3 (2.7- 107.6)	10	149.2 (2.0- 107.6)	10	38.9 (13.4- 249.4)	7	132.2 (42.3- 2406)	10	361457 (5188- 515805)	10	27.4 (5.2- 160.0)	9	688.9 (516.2- 997.7)
<i>p</i> -value		0.33		0.93		0.11		0.65		0.90		0.00		0.66		0.12
Vitamin C intake (mg)																
<EAR	39	1.3 (0.3- 2.1)	20	6.9 (1.3- 307.8)	20	46.9 (15.3- 371.7)	17	37.8 (16.5- 130.5)	17	261.5 (44.2- 941.8)	20	17714.9 (0- 812138)	19	17.5 (5.9- 235.5)	20	647.2 (158.8- 1787)
>EAR	134	1.4 (0.3- 2.1)	61	7.1 (1.4- 510.1)	52	54.9 (2.0- 377.9)	59	42.4 (8.0- 249.4)	50	136.4 (28.4- 2406)	59	21640 (0- 515805)	61	17.2 (3.9- 173.9)	58	588.1 (112.2- 1787)
<i>p</i> -value		0.07		0.85		0.87		0.61		0.42		0.80		0.23		0.62

Median (range). ^a, low vs. medium; ^b, low vs. high; ^c, medium vs. low; ^d, medium vs. high; ^e, high vs. low; ^f, high vs. medium; ^g, obese vs. normal; ^h, obese vs. overweight; ⁱ, <64.9 yr vs. 65.0-69.9 yr; ^j, <64.9 vs. >75 yr; ^k, 65.0-69.9 yr vs. >75 yr. Jonckheere trend test was applied. MDA, malondialdehyde; CAT Lmf, catalase in lymphocytes; SOD Lmf, superoxide dismutase in lymphocytes; GPX Lmf, glutathione peroxidase in lymphocytes; GRd Lmf, glutathione reductase in lymphocytes; MPO Plm, myeloperoxidase in plasma; CAT Plm, catalase in plasma; SOD Plm, superoxide dismutase in plasma; BML, body mass index; ER, retinol equivalents; EAR, estimated average requirements.

Table 24. Associations between oxidative stress parameters and health markers, age and dietary factors in females.

Variables	n	Co MDA (uM)	n	CAT Lmf (K/10 ⁹ Cells)	n	SOD Lmf (pKat/10 ⁹ Cells)	n	GPX Lmf (nkat/10 ⁹ Cells)	n	GRd Lmf (nkat/10 ⁹ Cells)	n	MPO Plm (nkat/mL)	n	CAT Plm (k/L)	n	SOD Plm (pkat/L)
Median (range)	229	1.4 (0.3- 25.1)	83	18.2 (1.1- 395.9)	73	3.7 (265.9)	77	38.3 (8.1- 555.9)	74	171.6 (26.7- 1887)	79	24686 (0- 720109)	79	22.0 (4.4- 295.6)	76	670.0 (148.8- 1850)
Age (yr)																
<64.9	80	1.3 (4- 5.0)	29	22.1 (1.1- 241.4)	24	32.0 (10.- 265.9)	26	33.8 (8.1- 202.8)	25	129.8 (37.0- 1273)	27	22759 (5192- 393052)	27	37.1 (.8- 295.6)	26	597.5 (148.8- 1169)
65.0-74.9	119	1.4 (4- 25.1)	44	12.0 (4- 395.9)	43	38.2 (3.7- 172.1)	42	40.7 (12.2- 555.9)	40	187.4 (26.7- 1032)	44	24533 (0- 720109)	44	16.5 (4.4- 150.4)	42	674.1 (212.6- 1850)
>75.0	30	1.3 (0.3- 22.2)	10	17.5 (2.5- 52.4)	6	42.6 (35.1- 76.1)	9	40.2 (17.4- 60.2)	9	192.4 (58.6- 1887)	8	27745 (10174- 349570)	8	21.5 (9.3- 201.5)	8	804.5 (486.2- 1144)
<i>p</i> -trend		0.00^{ij}		0.65		0.54		0.99		0.95		0.04^k		0.30		0.03^k
Physical fitness																
Low	72	1.6 (0.3- 25.1)	36	26.7 (1.1-395.9)	28	49.2 (3.7-260.2)	33	42.0 (10.6-322.5)	30	212.2 (34.7-1887)	30	33484 (5191- 470252)	33	24.2 (7.8- 295.6)	29	704.9 (280.9- 1850)
Medium	92	1.3 (0.4- 15.2)	32	6.1 (1.4-331.6)	31	30.2 (11.0-146.1)	29	30.6 (8.1-555.9)	30	127.7 (26.7-779.3)	33	15905 (4256- 393052)	33	19.9 (4.4- 201.5)	32	632.6 (148.8- 1170)
High	65	1.3 (0.5- 8.8)	15	11.6 (2.0-241.4)	14	44.7 (11.6-265.9)	15	34.6 (9.8-341.5)	14	191.6 (27.9-1273)	16	23131 (0-720109)	13	19.3 (7.1- 150.4)	15	628.8 (212.6- 1316)
<i>p</i> -trend		0.01^{ce}		0.04^e		0.56		0.29		0.38		0.05^e		0.37		0.87

Variables	n	Co MDA (uM)	n	CAT Lmf (K/10 ⁹ Cells)	n	SOD Lmf (pKat/10 ⁹ Cells)	n	GPX Lmf (nkat/10 ⁹ Cells)	n	GRd Lmf (nkat/10 ⁹ Cells)	n	MPO Plm (nkat/mL)	n	CAT Plm (k/L)	n	SOD Plm (pkat/L)
Physical activity and sedentary behaviour groups																
Inactive and high sedentarism	93	1.4 (0.4-22.2)	42	13.7 (1.1-237.8)	38	34.4 (10.3-265.9)	38	36.8 (8.3-555.9)	38	184.3 (34.7-1015)	40	24498 (3728-470253)	40	15.3 (4.4-138.0)	38	641.9 (148.8-1850)
Inactive and low sedentarism	67	1.3 (0.4-3.7)	15	23.0 (2.1-205.2)	13	68.1 (15.7-172.1)	15	46.4 (11.6-202.8)	11	199.5 (37.0-1887)	14	32836 (7755-308696)	14	42.4 (7.6-231.2)	14	735.4 (441.7-1009)
Active and high sedentarism	33	1.4 (0.4-18.4)	14	17.7 (1.8-331.6)	14	30.2 (3.7-93.3)	12	39.7 (8.1-341.5)	15	160.9 (58.6-779.3)	16	13505 (4256-308696)	14	32.0 (8.2-201.5)	16	616.9 (302.9-926.2)
Active and low sedentarism	36	1.5 (0.5-25.1)	12	19.0 (1.8-395.9)	8	64.9 (14.4-220.9)	12	30.1 (12.2-141.6)	10	337.6 (26.7-1273)	9	26555 (0-274290)	11	21.5 (13.6-295.6)	8	678.3 (541.8-1316)
<i>p</i> -trend		0.98		0.37		0.40		0.67		0.77		0.99		0.02		0.63
Smoking status																
Smoker	18	1.4 (0.6-15.2)	9	16.8 (2.1-177.9)	8	60.6 (15.7-265.9)	8	75.7 (10.6-279.5)	9	207.5 (37.0-790.8)	8	40856 (6991-278166)	9	31.2 (7.8-127.0)	8	722.7 (318.9-1378.6)
Non-smoker	210	1.4 (0.3-25.1)	74	18.5 (1.1-395.9)	65	35.1 (3.7-220.9)	69	37.7 (8.1-555.9)	65	165.3 (26.7-1887)	71	24378 (0-720109)	70	21.4 (4.4-295.6)	68	664.7 (148.8-1850)
<i>p</i> -value		0.64		0.93		0.06		0.22		0.94		0.25		0.66		0.56
BMI (kg/m²)																
Normal weight	83	1.4 (0.4-18.4)	30	17.7 (1.7-205.5)	28	35.6 (11.0-260.2)	30	32.6 (8.3-341.5)	27	207.5 (62.0-1887)	30	24652 (3728-720109)	31	27.0 (7.1-231.2)	30	720.2 (148.8-1379)
Overweight	100	1.4 (0.3-25.1)	40	21.3 (1.4-395.9)	34	41.5 (3.7-265.9)	36	40.0 (8.1-555.9)	36	181.2 (26.7-1273)	36	22106 (0-470252)	35	19.9 (4.4-295.6)	34	591.5 (233.8-1316)
Obese	46	1.4 (0.7-8.8)	13	3.6 (1.1-220.0)	11	38.2 (10.3-170.6)	11	41.2 (13.3-230.4)	11	142.3 (13.3-230.4)	13	25289 (4256-30869)	13	18.7 (6.0-138.0)	12	669.0 (212.6-1850)
<i>p</i> -trend		0.30		0.52		0.97		0.20		0.11		0.75		0.20		0.20

Variables	n	Co MDA (uM)	n	CAT Lmf (K/10 ⁹ Cells)	n	SOD Lmf (pKat/10 ⁹ Cells)	n	GPX Lmf (nkat/10 ⁹ Cells)	n	GRd Lmf (nkat/10 ⁹ Cells)	n	MPO Plm (nkat/mL)	n	CAT Plm (k/L)	n	SOD Plm (pkat/L)
WHtR																
Low WHtR (≤ 0.58)	171	1.4 (0.3- 25.1)	68	17.5 (1.4- 395.9)	61	36.1 (3.7- 265.9)	64	39.1 (8.1- 555.9)	62	171.6 (26.7- 1887)	65	22758 (0- 720109)	64	22.1 (4.4- 295.6)	63	655.1 (148.8- 1379)
High WHtR (≥ 0.58)	58	1.4 (0.5- 22.2)	15	18.7 (1.1- 220.0)	12	48.3 (10.3- 172.1)	13	37.7 (13.3- 230.4)	12	176.6 (37.0- 649.7)	14	39356 (4256- 430217)	15	19.3 (8.2- 138.0)	13	704.9 (212.6- 1850)
<i>p</i> -value		0.31		0.63		0.72		0.64		0.99		0.07		0.77		0.58
Alcohol (ml/day)																
0	226	1.4 (0.3- 25.1)	82	18.5 (1.1- 395.9)	72	37.1 (3.7- 260.2)	76	38.0 (8.1- 555.9)	73	117.8 (26.7- 1887)	78	24652 (0- 720109)	78	22.1 (4.4- 295.6)	75	671.3 (148.8- 1850)
0.1-149.9	1	1.4														
>150.0	2	2.2 (1.7- 2.7)	1	16.8	1	265.9	1	142.8	1	84.5	1	278167	1	11.9	1	318.9
<i>p</i> -trend		0.18		0.97		0.09		0.21		0.44		0.15		0.48		0.14
Fruit																
Once a day	32	1.3 (0.4- 2.2)	8	11.0 (3.4- 205.5)	8	45.0 (13.2- 265.9)	7	41.4 (24.9- 230.4)	8	122.7 (54.7- 489.4)	8	15962 (4256- 72019)	8	30.1 (8.8- 150.4)	8	648.6 (302.9- 859.8)
2-3/day	133	1.4 (0.3- 25.1)	47	12.4 (1.1- 395.9)	41	31.4 (3.7- 260.2)	46	37.6 (8.3- 555.9)	41	142.3 (26.7- 1032)	44	27570 (6095- 470253)	46	21.9 (4.4- 295.6)	42	673.9 (148.8- 1850)
>4/day	62	1.4 (0.4- 8.8)	28	29.3 (1.8- 331.6)	24	45.8 (12.8- 220.9)	24	41.8 (8.1- 279.5)	25	216.8 (34.7- 1887)	27	22793 (0- 393053)	25	22.0 (6.1- 201.5)	26	678.1 (212.6- 1379)
<i>p</i> -trend		0.42		0.04		0.58		0.90		0.30		0.85		0.84		0.71

Variables	n	Co MDA (uM)	n	CAT Lmf (K/10 ⁹ Cells)	n	SOD Lmf (pKat/10 ⁹ Cells)	n	GPX Lmf (nkat/10 ⁹ Cells)	n	GRd Lmf (nkat/10 ⁹ Cells)	n	MPO Plm (nkat/mL)	n	CAT Plm (k/L)	n	SOD Plm (pkat/L)
Selenium intake (µg)																
<EAR	17	1.5 (0.4- 4.6)	9	26.3 (2.4- 331.6)	8	46.5 (14.4- 170.6)	6	46.0 (11.6- 176.4)	8	255.9 (27.9- 990.3)	8	20237 (7618- 720109)	9	69.8 (7.6- 295.69)	8	647.1 (495.0- 856.4)
>EAR	210	1.4 (0.3- 25.1)	74	16.8 (1.1- 396.8)	65	36.1 (3.7- 265.9)	71	37.7 (8.10- 555.9)	66	155.7 (26.7- 1887)	71	24907 (0- 470253)	70	21.4 (4.4- 231.2)	68	673.9 (148.8- 856.4)
p-value		0.96		0.22		0.27		0.96		0.52		0.39		0.12		0.95
Vitamin A intake (ER)																
<EAR	64	1.3 (0.3- 25.1)	17	20.5 (2.0- 395.9)	14	35.6 (11.6- 105.0)	16	29.6 (17.4- 230.4)	17	94.1 (27.9- 1887)	14	18395 (4256- 35878)	17	24.4 (9.0- 201.5)	14	567.3 (302.9- 859.8)
>EAR	163	1.5 (0.4- 18.4)	66	17.5 (1.1- 331.6)	59	42.0 (3.7- 265.9)	61	41.2 (8.1555.9)	57	190.8 (26.7- 1273)	65	26555 (0- 720109)	62	21.4 (4.4- 295.6)	62	676.6 (148.8- 1850)
p-value		0.00		0.59		0.82		0.24		0.78		0.04		0.45		0.30
Vitamin E intake (mg)																
<EAR	218	1.4 (0.34- 25.1)	80	17.5 (1.1- 395.9)	70	39.2 (3.7- 265.9)	74	38.0 (8.1- 55.9)	72	184.3 (26.7- 1887)	76	25098 (3728- 720109)	78	21.8 (4.4- 295.6)	73	671.3 (148.8- 1850)
>EAR	9	1.1 (1.0- 1.7)	3	28.2 (6.5- 30.3)	3	22.0 (13.3- 88.3)	3	43.4 (25.8- 46.8)	2	84.2 (80.4- 88.1)	3	5191 (0- 22429)	1	27.6	3	358.3 (320.3- 1316)
p-value		0.00		0.68		0.76		0.98		0.23		0.17		0.73		0.53
Vitamin C intake (mg)																
<EAR	20	1.3 (0.3- 4.6)	10	24.7 (3.6- 205.5)	9	48.1 (13.2- 159.0)	9	41.4 (12.2- 230.4)	9	239.9 (65.5- 990.3)	9	24686 (4256- 720109)	10	71.9 (9.0- 295.6)	9	592.8 (302.9- 859.8)
>EAR	207	1.4 (0.4- 25.1)	73	16.8 (1.1- 395.9)	64	35.6 (3.7- 265.9)	68	37.6 (8.1- 555.9)	65	165.3 (6.7- 1887)	70	24762 (0- 47053)	69	21.3 (4.4- 231.2)	67	676.8 (148.8- 1850)
p-value		0.59		0.33		0.34		0.73		0.41		0.48		0.06		0.21

Median (range). ^a, low vs. medium; ^b, low vs. high; ^c, medium vs. low; ^d, medium vs. high; ^e, high vs. low; ^f, high vs. medium; ^g, obese vs. normal; ^h, obese vs. overweight; ⁱ, <64.9 yr vs. 65.0-69.9 yr; ^j, <64.9 vs. >75 yr; ^k, 65.0-69.9 yr vs. >75 yr. Jonckheere trend test was applied. MDA, malondialdehyde; CAT Lmf, catalase in lymphocytes; SOD Lmf, superoxide dismutase in lymphocytes; GPX Lmf, glutathione peroxidase in lymphocytes; GRd Lmf, glutathione reductase in lymphocytes; MPO Plm, myeloperoxidase in plasma; CAT Plm, catalase in plasma; SOD Plm, superoxide dismutase in plasma; BMI, body mass index; ER, retinol equivalents, EAR, estimated average requirements.

Tables 25-26 display the linear regression model between oxidative stress parameters and lifestyles variables in each sex. As shown Table 25, MPO was strongly relationship with increase vitamin E (standardized $\beta=0.44$, $p<0.001$) and decrease age (standardized $\beta= -0.34$, $p<0.01$) in model 2, explaining 28% of the variance. Furthermore, SOD Lmf was strongly associated with increase PF level (standardized $\beta =0.39$, $p<0.001$).

Table 25. Linear regression analysis of the association between oxidative stress parameters with lifestyle variables in males.

MDA (uM) (n=175)		β	R ²	p
Model 1				
	Vitamin C (mg)	0.16	0.03	0.03
CAT Lmf (K/10^9 Cells) (n=81)				
Model 1				
	BMI (kg/m ²)	0.29	0.07	<0.01
SOD Lmf (pKat/10^9 Cells) (n=72)				
Model 1				
	Physical fitness	0.39	0.14	<0.001
GPX Lmf (nkat/10^9 Cells) (n=76)				
Model 1				
	Vitamin A (ER)	0.34	0.10	<0.01
MPO Plm (nkat/mL) (n=79)				
Model 1				
	Vitamin E (mg)	0.43	0.17	<0.001
Model 2				
	Vitamin E (mg)	0.44	0.28	<0.001
	Age (yr)	-0.34		<0.01
SOD Plm (pkat/L) (n=78)				
Model 1				
	Selenium (μg)	0.25	0.05	0.03

β , standardized regression coefficients. MDA, malondialdehyde; CAT Lmf, catalase in lymphocytes; SOD Lmf, superoxide dismutase in lymphocytes; GPX Lmf, glutathione peroxidase in lymphocytes; MPO Plm, myeloperoxidase in plasma; SOD Plm, superoxide dismutase in plasma; BMI, body mass index. Level of significant $p<0.05$.

In females (Table 26), MDA was negative associated with physical fitness level (standardized $\beta = -0.16$, $p=0.02$) and also CAT Plm showed a negative relationship with vitamin E (standardized $\beta = -0.24$, $p=0.03$). Meanwhile, SOD Lmf was positive linked with alcohol (standardized $\beta = 0.26$, $p=0.03$) and SOD Plm with age (standardized $\beta = 0.26$, $p=0.03$).

Table 26. Linear regression analysis of the association between oxidative stress parameters with lifestyle variables in females.

MDA (uM) (n=229)		β	R^2	p
Model 1				
	Physical fitness	-0.16	0.02	0.02
SOD Lmf (pKat/10 ⁹ Cells) (n=73)				
Model 1				
	Alcohol (mL/day)	0.26	0.06	0.03
CAT Plm (k/L) (n=79)				
Model 1				
	Vitamin E (mg)	-0.24	0.05	0.03
SOD Plm (pkat/L) (n=76)				
Model 1				
	Age (yr)	0.26	0.05	0.03

β , standardized regression coefficients. MDA, malondialdehyde; CAT Lmf, catalase in lymphocytes; SOD Lmf, superoxide dismutase in lymphocytes; GPX Lmf, glutathione peroxidase in lymphocytes; GRd Lmf, glutathione reductase in lymphocytes; MPO Plm, myeloperoxidase in plasma; CAT Plm, catalase in plasma; SOD Plm, superoxide dismutase in plasma; BMI, body mass index. Level of significant $p < 0.05$.

4.5.5 Discussion

The present investigation revealed important data about the relationship between oxidative stress parameters and lifestyle behaviours. The associations between PA and PF with oxidative stress biomarkers are still controversial (382). Indeed, Bouzid et al. indicated that both low and high PF levels help to maintain better antioxidants defences in older adults. Nevertheless, higher PF level could rise lipid peroxidation rather than low PF level (50). Our main results showed that sex should be considered in the analysis between oxidative stress parameters and lifestyle factors. Additionally, age presented association with several oxidative stress parameters such a MDA, CAT, SOD Lmf and MPO. Aging has been related to an increase oxidant generation and an accumulation of end products of oxidative damage (156,204,309). Unexpectedly, a positive and significant association was found between SOD Lmf and age in females, whereas negative and significant relationship was observed between MPO and age in males. Andersen et al. observed that an age reduced SOD concentration and glutathione reductase activity, together with no changes in the activities of GPx and CAT with increasing age (14). Regarding the antioxidant enzyme activity, Mahasned et al. indicated that a strong positive link was observed with total antioxidant capacity and age (204). These differences could be due to the small sample size of participants aged over 75 yr in our study.

Regular PA at moderate intensity may induce favourable changes in antioxidant defence system, especially by an increase of individual enzymatic antioxidants activity (124). However, Fraile-Bermúdez et al. observed that total antioxidant capacity was lower in the more active groups measured by means of accelerometer (116). Simultaneously, Fraile-Bermúdez et al. found that moderate to vigorous PA (MVPA) was correlated with increase in the GPx antioxidant enzyme activity, and the counts per minute (cpm) were positively correlated with CAT activity (116). The MVPA and cpm were negatively correlated with lipid peroxidation whereas PA was positively correlated with CAT activity in males (116).

Also, Wu et al. showed that greater levels of PA were related to a decreased of SOD activity (382). Likewise, PA was not related to GPx and CAT activities after adjusting for covariables (382). CAT Plm showed a relationship with PA and sedentarism groups which extreme groups presented lower levels of CAT Plm activity than the other PA and sedentarism group (193). Mahasneh et al. did not observe any association with

regular PA in females (204). In our study, we found that higher levels of PF were related to SOD Lmf activity in males and a negative association was observed between MDA and PF in females. Excess level of MDA levels has been reported when it undergoes cell damage or in several diseased conditions. Several authors are in concordance with our results (192,193,261). Rowinski et al. found that oxidative stress parameters increase was age-related; nonetheless PA can decrease oxidative stress markers and induce adaptive increase in the erythrocyte antioxidant enzyme activity, mainly SOD, even in old and very old males and females (301).

Our results showed that higher MPO activity was associated with high WHtR in females; nevertheless, CAT Lmf activity was positive related to obese male. In line with our results, Mahasneh et al. found that MPO activity increased with high BMI although GPx was negative related to BMI in females (204). Greater oxidative stress is linked with stored body fat, chiefly visceral obesity related to metabolic syndrome (157).

Regarding with negative lifestyle behaviour such as smoking habits, SOD Lmf showed difference between smoker and non-smoker, being higher levels of SOD Lmf smoker female's participants. There were several studies which found this association (232,263). On the contrary, others authors observed an inverse relationship between SOD and smokers (204,254). Comparisons between alcohol intake and oxidative stress parameters were difficult in our study due to the small number of participants who drank frequently alcohol. The regression model showed that higher SOD Lmf activity increased with alcohol consumption (beer, wine and distilled drink). Fernández-Pachon et al. observed that the consumption of daily red wine decreased SOD, CAT and Gr activities (108). Lesgards et al. showed a negative relationship with total antioxidant activity (193). However, Mahasneh et al. found that alcohol consumption was related to greater GPx activity (204).

Greater antioxidants intakes, principally vitamin C, appear to be associated with higher skeletal muscular strength in elderly persons. The body contains an elaborate antioxidant defence, vitamin C, vitamin E, carotenes, and retinol are the primary antioxidant vitamins (66). In this sense, we observed a positive association between MDA with vitamin C, GPx with vitamin A, MPO with vitamin E, and SOD with selenium in males. In females, CAT showed a negative association with vitamin E. Likewise, we found that CAT Lmf was associated with higher intake of fruits per day in females. The results obtained are in concordance with others authors (148,192,204),

nevertheless, the regression model showed an inverse relationship between vitamin E intake and CAT Plm. This may be due to a considerable number of participants presented intake below EAR.

Limitations and strengths

This study has several limitations. Firstly, the cross-sectional design of this study does not allow drawing any conclusions regarding causation. Moreover, the sample consisted of people who participated voluntarily. Secondly, the small sample size is limited in this study. Despite of the above-mentioned limitations, the study has several strengths. One of the strengths of our study was the sampling procedure and the strict standardization of the field work among the cities involved in the study avoided to a great extent the kind of confounding bias. Another strength is the association between oxidative stress parameters and several lifestyle behaviours which studies are scarce in Spanish older adults.

4.5.6 Conclusions

Different behaviours were found between sexes and oxidative stress parameters. Our results also indicate that some oxidative stress parameters are related to lifestyle behaviours. Several associations were displayed between oxidative stress parameters and PF, age, antioxidant intake and body composition. Indeed, higher lipid peroxidation measured through MDA was found in the high PF level in females, whereas higher antioxidant enzyme activity measured by SOD was observed in the high physical fitness level in males. Further longitudinal studies are needed in order to elucidate these associations.

4.6 ARTICLE 6: WHICH COMMON BLOOD MARKERS COULD BE USED TO DEFINE PHYSICAL FITNESS STATUS IN OLDER ADULTS?

Aparicio-Ugarriza R, Díaz E, Palacios G, Bibiloni MM, Julibert A, Tur JA, González-Gross M. J Clin Lipidol (submitted). (JCR: 5.812).

4.6.1 Abstract

Biomarkers have been postulated as the as the essential variables to measure the effect of exercise on the human body. To investigate the relationship between physical fitness (PF) levels with blood biomarkers which are associated to disease risk in Spanish older adults. **Methods:** Four hundred twenty-nine adults (57% females) from a cross-sectional multicentre study, aged over 55 yr were included. A battery of PF test was performed and participants were divided into 3 levels: low, medium and high. Blood samples were collected and participants were also grouped depending if a particular biomarker was out or in of its reference range. Furthermore, drug intake was considered from each participant. **Results:** Higher values out of reference range were observed for vitamin 25[OH]D (67.9%) and TC (58.6%). Subjects from the low PF group presented lower significant concentrations out of the reference for vitamin B₁₂ and triglycerides; however, subjects in the low PF group showed higher significant abnormal values for total homocysteine, creatinine, total cholesterol (TC), HDL-cholesterol and LDL-cholesterol (LDL-c) than in the high PF group (all $p < 0.05$). Considering drugs related to blood lipid modifications, subjects who regularly consumed lipid-reducing presented higher significant values out of the reference range for TC and LDL-c than participants who did not take these drugs ($p < 0.01$). **Conclusions:** Blood markers of health were associated with PF status in older adults. Those presenting a better blood markers profile had high PF. These markers could be used as a routine method to assess PF levels in this kind of population. Also, drug intake should be considered.

Key words: physical fitness; biomarkers; health status indicators; aging.

4.6.2 Introduction

There is an increasing need to evaluate health-related aspects (i.e. biomarkers) which can be modified by regular physical exercise (258) in order to provide evidence-based strategies for healthy aging (158). Aging phenomena is linked with an increasing risk for cognitive dysfunction (282) and chronic diseases (12), among others. Both factors contribute to changes in motor functions and impairments in physical performance in the old population. These changes could have an effect in the ability of older adults to carry out the daily activities (319).

The pro-healthy effects of regular physical activity (PA) are well-documented (35,333) and previous studies have revealed that physical fitness (PF) is a crucial independent predictor of mortality (43,178). Attributable risk estimates for all-cause mortality showed that low PF level, principally low cardiorespiratory fitness and low muscular strength, is a significant risk factor in both sexes (43,305). PF mixes the majority of the body functions which are involved in the performance of daily PA (256). Decreases in PF level are related to several negative health effects (355) and biomarkers have been proposed as essential markers to evaluate the effect of exercise on human body (49,258).

Vitamin deficiencies can cause disorders in behavior, cognitive and emotional status, and personality (133). Concretely, epidemiologic data indicates the prevalence deficiency of vitamin B₁₂ from 6% to 40% (21). Additionally, the worldwide prevalence of pernicious anaemia in elderly (aged over 65 yr) has been estimated to be 23.9% (222). B vitamins and high total homocysteine (tHcy) concentrations have been connected with CVD (especially stroke) (155,250), cognitive decline and dementia (133,283,327), fractures (222,327) and mortality (75,384). Likewise, low vitamin D status may stimulate adipogenesis (82), resulting in further increases in adiposity. Low vitamin D concentrations are also linked with muscle pain, muscle weakness (153,365) and poor PF in older adults (7).

Blood lipid profile also affects the progress of coronary heart disease (168). Furthermore, with increasing age, some changes in lipid and lipoprotein concentrations are overall unfavourable (265).

Through the analysis of different biomarkers, it could be possible to evaluate the reaction of the human body at the diverse levels of PF (258). However, research about the relationship between biomarkers and PF in the old adult population is still ongoing.

Hence, the main purpose of this study was to investigate if some blood biomarkers associated to disease risk could be used for PF assessment depending on their blood concentrations in Spanish older adults.

4.6.3 Materials and Methods

Study design, sample and ethics

The PHYSMED project is a multi-centre cross-sectional study aiming at identifying cardiovascular risk factors in sedentary and active older participants. Data were collected from April 2013 to May 2014 and field work took place in 2 Spanish cities: Madrid and Mallorca. Participants were recruited through health centres, sport federations, sport facilities and municipal clubs located at Madrid and Mallorca following snowball system. The study population included 429 adults (57% females), aged 55-88 yr. Individual exclusion criteria were: age under 55 yr, being institutionalized, suffering from a physical or mental illness which would have limited their participation in the PF tests or their ability to respond to the questionnaires or drug intake for clinical research.

The study followed the Declaration of Helsinki 1964 and further amendments. Also, the protocol study was approved by the Ethical Committee of the Universidad Politécnica de Madrid. Each participant signed a written informed consent prior to his/her participation.

Specimen collection and biochemical analyses

Fasted (12h) blood samples were collected from each participant, and lipid profile and basic biochemistry were analysed at the biochemical laboratories of the High Sports Council (Madrid) and Hospital Son Espases & University of Balearic Islands (Mallorca). The extraction was performed by standard venepuncture on vacuum Vacutainer® tubes with separation gel for serum or EDTA for anticoagulant. Gel for serum tubes were immediately placed on ice and after coagulum formation the sample was centrifuged at 3,000 rpm for 10 min. Serum total cholesterol (TC), HDL-cholesterol (HDL-c), LDL-cholesterol (LDL-c), triglycerides (TG), glucose, urea, uric acid, total protein, albumin, glutamic oxalacetic transaminase, glutamic-pyruvic transaminase, gamma glutamyl transpeptidase, iron (FE) and ferritin (FER) were analysed in fresh serum using the Beckman AU400 analyser (Beckman AU400, Beckman Instruments,

Ltd., Bucks, UK) by photometric methods. Creatinine was analysed by colorimetric method (Beckman AU400, Beckman Instruments, Ltd., Bucks, UK). Likewise, haematocrit and hemoglobin analysis were performed within the first hour after extraction in EDTA tubes using an automated hematology analyser (ADVIA 120 (Siemens Health Care Diagnostics, SA). These determinations were carried out in each site.

Vitamin B₁₂, serum folate (sfolate), red blood cell folate (RBC folate), vitamin 25[OH]D and tHcy analyses were centralized at the High Sports Council Laboratory in Madrid. RBC folate was measured using EDTA tubes. Therefore, serum samples from Palma de Mallorca were dry-ice shipped to Madrid and analysed together with those serum samples stored in Madrid at -80°C, by electrochemoluminescence method (Elecsys 2010, Roche Diagnostics, Switzerland), except vitamin 25[OH]D which was determined using E411 analyser (Roche Diagnostics, Switzerland).

Physical fitness tests

Prior to assess PF tests, blood pressure was measure using digital monitor (Omron Healthcare Co., Ltd. Kyoto, Japan). Each participant completed a multi-component battery of PF test validated in the elderly population (290) and validated reference ranges for Spanish older adults proposed by Pedrero-Chamizo et al. (269). Lower body strength was measured by the chair stand test (290), agility/dynamic balance was performed by the 8-foot up-and-go test (290), aerobic endurance was assessed by the 6-min walk test (290) and handgrip strength was measured with a handgrip dynamometer (361) (Takei TTK 5401, Tokyo, Japan, range 5-100 kg, precision 0.1 kg). The handgrip strength was assessed for both hands in a standing position. All tests were performed twice, except the 6-min walk test and the chair stand test, and the best score was retained.

The results of each PF tests were stratified by sex and five age groups (divided by five years periods except the last group) following the criteria established by Pedrero-Chamizo et al. (269). Also, the result of each PF test was divided into quartiles considering the variables above mentioned (sex and age groups). The score for each test ranged from 0 (worst) to 3 (best) points. Thus, the maximum score was 12 points. Scores of PF tests were added together to create a cluster. After that, PF cluster analyses were performed and each cluster was divided into different levels. In order to classify

our population taking into account analysis of clustering, we established three groups of PF: low, medium and high.

Anthropometric measurements

Weight, body mass index (BMI), total body water (TBW) and fat free mass (FFM) were measured using bioimpedance analysis (TANITA Corp, BC-418MA, Tokyo, Japan) in standardized conditions. Also, BMI was calculated as the weight (kg) divided by the square of the height (m²). Height was assessed to the nearest millimetre using a mobile stadiometer (SECA 213, Germany), with the participant's head in the Frankfurt plane.

Dietary assessment

Dietary intake was obtained by means of two 24h dietary recalls collected on two non-consecutive days within a period of 2-3 weeks by well-trained dieticians. A computer program was used to convert food into nutrients (ALIMENTA®; NUCOX, Palma, Spain) based on Spanish (218,255) and European (106) food composition tables, and complemented with food composition data available for Mallorca food items (291).

Socioeconomic and lifestyle questionnaire

A general EXERNET questionnaire was used including smoking habits. Participants were grouped in categories as follows: (i) educational level: primary school, secondary school and college-level education; (ii) current income: <600 €/month, 600-900 €/month and ≥900 €/month; (iii) smoker (≥1 cigarette/day) and non-smoker.

Medication intake

Participants were asked by means of the EXERNET questionnaire according to the following question: Do you regularly take drugs? (Yes/No). If they answered Yes, they were asked about type of medication, manufacturer, frequency of consumption and dose of each drug. After that, each drug was coded following the Spanish Agency of Medicines and Sanitary Products (AEMPS, Medicines Online Information Center-CIMA) (<https://www.aemps.gob.es/cima/fichasTecnicas.do?metodo=detalleForm>). Four groups of drug intake were considered: antidiabetic, renin-angiotensin system, beta blocker and lipid-lowering drugs and all variables were grouped if participants took (coded as 1) or not took each of the drugs (coded as 0).

Statistical analysis

Statistics were carried out using statistical software SPSS (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp). Descriptive characteristics were summarized by calculating means and standard deviations (SD) unless otherwise stated. Each variable was checked for normality of distribution using the Kolmogorov-Smirnov test. The urea, uric acid, total protein, albumin, TC, HDL-c, LDL-c, haemoglobin, haematocrit and vitamin 25[OH]D values showed normal distribution. The differences between descriptive characteristics and sexes and the differences between biomarkers and PF groups were performed using One-way ANOVA (for normally distributed variables) and Kruskal-Wallis test (for non-normally distributed variables). *Post hoc* analyses were conducted with the Bonferroni adjustment. All variables of PF tests were checked for normality of distribution by Kolmogorov-Smirnov test and these presented normal distribution.

Regarding vitamin D, the season and the date of blood sample collection was considered for statistical analysis. Samples collected from October to March were classified as “autumn-winter samples” and those collected from April to September were classified as “spring-summer samples”. The latitudes of each city are similar: Palma de Mallorca (39° 34” N) and Madrid (40° 24” N).

Variables were categorized as being within reference range (coded as 1) or out of the reference range (coded as 0). Reference ranges which have been used were those established by each laboratory for adults obtained from population served (see Article 7). Punctually, for vitamin B₁₂ and related biomarkers different cut-off has been applied (21).

The association between the status of each biomarker and PF groups was assessed using the generalized lineal model. Each biomarker coded as 0 (out of the reference range) was used as reference in this statistical model. PF groups were used as independent variable in which the high PF group was considered as the reference. Depending on the biomarkers, drug intake was included into the model as independent variable in which drug intake was the reference. Moreover, several diet covariables were considered depending on the biomarkers analysed. The model was not considered when the level of significance of the omnibus test was higher than 0.05. In turn, the lower Akaike information criterion was chosen in the final models. Albumin was not analysed by

means of the generalized linear model because only one participant presented abnormal concentrations. Values of $p < 0.05$ were considered statistically significant.

4.6.4 Results

Table 27 includes the participants characteristics split by PF groups.

Table 27. Descriptive characteristics of the studied sample split by physical fitness groups.

	Physical fitness groups			p-value
	Low (n=131)	Medium (n=172)	High (n=126)	
	Mean±SD Median (min-max)	Mean±SD Median (min-max)	Mean±SD Median (min-max)	
Sex (female)	76 (58.6)	98 (56.9)	70 (55.6)	>0.05
Age (yr)	66.8±7.1 66.0 (55.0-87.8)	66.6±6.6 66.0 (55.0-85.4)	66.5±6.2 66.0 (55.1-80.8)	>0.05
City (Madrid)	30 (22.6)	83 (47.7)	87 (69.0)	>0.05
Height (cm)	161.2±8.8 159.5 (143.0-183.0)	162.2±9.2 160.0 (142.0-184.0)	163.9±8.9 164.0 (145.5-185.5)	<0.05 ^b
Weight (kg)	72.4±13.5 70.3 (44.9-116.6)	72.9±12.9 71.5 (44.0-99.5)	69.1±11.4 68.2 (46.1-104.6)	<0.05 ^c
BMI (kg/m ²)	27.8±3.6 27.4 (17.1-39.1)	27.7±4.1 27.5 (18.4-41.8)	25.6±3.1 25.5 (17.0-33.4)	<0.001 ^{a,b,c}
Energy (kcal/day)	1666±486 1618 (540.7-3121)	1650±438 1603 (722-3154)	1778 ±492 1722 (884-3515)	<0.01 ^b
Smoker	16 (12.0)	6 (3.5)	12 (9.5)	<0.05 ^a
Education				
Primary school	78 (58.6)	30 (22.6)	25 (18.8)	<0.05 ^{a,b,c}
Secondary school	68 (39.9)	57 (34.1)	45 (26.0)	
University graduate	40 (31.8)	41 (32.5)	45 (35.7)	
Current income				
<600 €/month	43 (32.3)	19 (14.3)	71 (53.4)	<0.001 ^{b,c}
600-900 €/month	41 (24.1)	23 (13.5)	106 (62.4)	
>900 €/month	11 (8.7)	17 (13.5)	98 (77.8)	
Systolic blood pressure (mmHg)	141.0±18.3 138.8 (93.5-219.5)	140.3±17.6 139.3 (100-213.5)	139.6±17.2 139.0 (104.5-185.5)	>0.05
Diastolic blood pressure (mmHg)	81.0±9.8 81.0 (54.5-108.5)	79.4±10.4 79.5 (50.0-126.0)	77.5±8.1 76.5 (58.0-102.0)	<0.05 ^a
Waist perimeter (cm)	91.6±10.9 91.4 (67.0-121.3)	90.2±11.5 89.9 (61.3-113.7)	86.2±11.2 85.2 (62.7-124.7)	<0.001 ^{b,c}
Hip perimeter (cm)	102.6±8.1 102.0 (82.7-140.0)	102.5±8.3 102.0 (84-136.8)	99.2±6.7 99.1 (84.0-121.8)	<0.001 ^{b,c}

Data are presented as means $\pm$ SD; median (minimum-maximum); n, no.of participants and values (%). BMI, body mass index. Comparisons between physical fitness groups were analyzed by One Way ANOVA or Kruskal- Wallis test, according to the normality of the variables. *Post hoc* analyses were conducted with the Bonferroni adjustment. ^a: Low fitness vs. medium fitness; ^b: low fitness vs. high fitness; ^c: medium fitness vs. high fitness. Level of significance $p<0.05$.

The descriptive data of biomarkers according to PF groups are shown in Table 28. Creatinine ($p<0.001$), TC ($p<0.05$), HDL-c ($p<0.001$), LDL-c ($p<0.01$), sfolate ($p<0.001$) and RBC folate ($p<0.01$) were significantly higher in the high PF group than in the low and medium PF groups. Those with high PF level also showed lower significant differences for total protein and TG concentrations than those who had low and medium PF level (both $p<0.05$).

Table 28. Descriptive data of biomarkers divided by physical fitness groups.

Biomarkers	Physical fitness groups					
	Low		Medium		High	
	n	Mean±SD Median (min-max)	n	Mean±SD Median (min-max)	n	Mean±SD Median (min-max)
Glucose (mg/dL)	129	99.5±15.7 96.0 (75.0-190.0)	171	99.1±12.7 98.0 (75.0-144.0)	126	98.1±14.8 97.0 (71.0-181.0)
Urea (mg/dL)	130	36.3±9.3 35.9 (19.0-69.0)	171	37.5±8.2 37.0 (20.0-59.8)	126	37.5±8.2 37.0 (17.7-62.2)
Uric acid (mg/dL)	130	5.4±1.3 5.1 (2.8-9.4)	171	5.4±1.4 5.5 (2.6-9.4)	126	5.3±1.3 5.2 (3.0-8.9)
Creatinine (mg/dL)	131	0.82±0.21 0.78 (0.53-2.18)	172	0.86±0.17 0.84 (0.57-1.46)	126	0.93±0.17 0.91 (0.64-1.41)
Total protein (g/L)	62	70.4±3.6 70.0 (60.5-81.0)	116	70.3±3.9 70.0 (62.0-81.0)	97	69.0±3.7 69.0 (60.0-78.0)
Albumin (g/L)	61	42.2±2.1 42.0 (37.7-46.0)	116	42.1±2.1 42.0 (37.0-49.0)	97	42.0±2.5 42.0 (35.0-48.0)
TC(mg/dL)	130	204.0±35.3 198.5 (112.0-293.0)	172	210.8±34.6 206.0 (141.0-351.0)	126	218.4±33.3 219.0 (131.0-321.0)
HDL-c (mg/dL)	129	53.0±12.6 53.0 (20.0-91.0)	169	55.8±13.1 54.5 (27.7-95.2)	124	59.2±13.5 57.9 (32.6-98.0)
LDL-c (mg/dL)	129	129.7±30.1 126.0 (69.4-218.8)	169	135.1±29.6 134.0 (69.0-249.5)	124	141.3±27.4 142.7 (71.4-224.8)
TG (mg/dL)	130	104.3±38.4 97.2 (33.0-208.0)	172	99.1±40.8 90.1 (45.2-268.0)	126	93.9±37.9 84.1 (40.6-274.1)
Haematocrit (%)	131	43.4	168	43.3	125	43.1
Hemoglobin (g/dL)	131	14.6±1.2 14.7 (12.0-17.0)	168	14.5±1.1 14.3 (12.0-18.0)	125	14.5±1.0 14.6 (12.0-17.0)
FE (ug/dL)	61	93.1±23.8 94.0 (38.2-137.5)	115	91.6±33.9 87.5 (38.3-331.0)	98	93.8±28.2 90.7 (45.4-201.2)
FER (ng/dL)	60	122.3±101.0 110.7 (12.0-671.5)	115	125.5±99.5 93.6 (13.0-542.0)	96	126.6±93.4 98.0 (16.8-419.3)
tHcy (μmol/dL)	111	13.4±4.6 12.8 (1.8-35.5)	164	12.9±4.4 12.2 (2.0-31.3)	124	12.3±4.0 11.6 (3.9-34.2)

	Low			Medium			High		
	n	Mean±SD Median (min-max)	n	Mean±SD Median (min-max)	n	Mean±SD Median (min-max)	n	Mean±SD Median (min-max)	p-value
Vitamin B₁₂ (pg/ mL)	109	375.8±181.2 345.3 (86.5-1163)	163	390.3±202.0 343.7 (122.8-1650)	124	386.4±172.4 350.2 (100.0-1402)			>0.05
sFolate (ng/ mL)	111	10.1±4.2 8.8 (2.7-32.7)	164	11.1±4.4 10.4 (3.9-29.3)	124	11.9±4.2 11.7 (4.7-29.9)			<0.001
RBC folate (ng/ mL)	91	345.9±117.2 326.9 (156.1-728.8)	133	369.2±114.5 351.2 (184.2-862.6)	113	384.2±105.5 379.1 (149.0-710.0)			<0.01
Vitamin D(25(OH)D) (mg/mL)	109	25.6±10.3 24.1 (4.5-51.9)	154	25.9±9.8 25.4 (4.3-64.4)	120	26.0±10.5 25.5 (6.7-57.7)			>0.05

Values are presented as means±SD; n, median (minimum-maximum); no. of subjects. sFolate, serum folate; RBC folate; red blood cell folate; tHcy, total homocysteine; TC, total cholesterol; TG, triglycerides; HDL-c, HDL-cholesterol; LDL-c, LDL-cholesterol; FE, iron, FER, ferritin. Comparisons between physical fitness groups were analyzed by One Way Anova or Kruskal- Wallis test, according to the normality of the variables. Level of significance p<0.05.

Figure 23 displays the percentage of each biomarker in and out of the reference range. A total of 67.9% and 58.6% of the sample presented vitamin 25[OH]D deficiency and excessive TC, respectively. Higher values were obtained for glucose (38.3%), uric acid (32.3%), LDL-c (27.7%) and tHcy (24.3%).

Values are presented as percentage (%). RBC folate, red blood cell folate; sFolate, serum folate; tHcy, total homocysteine; TC, total cholesterol; TG, triglycerides; HDL-c, HDL-cholesterol; LDL-c, LDL-cholesterol; FE, iron, FER, ferritin.

Figure 23. Percentage of each biomarker in and out the reference range.

Results from the general linear model investigating the associations between biomarkers and PF groups are shown in Table 29. Participants from the low PF group were associated with higher vitamin B₁₂ levels within reference range (both models, $p < 0.05$). However, belonging to the low PF group showed greater tHcy concentrations out of reference range than high PF group (both models, $p < 0.05$). Furthermore, with advanced age (in male) vitamin B₁₂ deficiency and tHcy levels increased in parallel significantly ($p < 0.05$). Likewise, high total protein concentrations out of range were observed in the low PF group (both models, $p > 0.05$) and medium PF group (both model, $p > 0.05$) in comparison to the high PF group. Participants in the low PF group also showed higher possibility to have creatinine levels out of the reference range than those high PF group (both models, $p < 0.05$).

Concerning the lipid profile, more participants included in the low PF group were out of range for TC than those in the high PF group (both models, $p < 0.001$). Males presented more concentrations of TC than females ($p < 0.001$). Additionally, when taking into

account lipid and fatty acids intake in the model, these associations remained ($p < 0.05$). Similarly, participants from the low (both models, $p < 0.001$) and medium (both models, $p < 0.05$) PF groups presented greater HDL-c levels out of reference range than high PF group. Likewise, people showing low PF had low risk of high LDL-c concentrations than high PF group (model 2, $p < 0.05$). Taking into account drugs related to blood lipid modifications, participants who regularly consumed lipid-reducing drugs presented higher values out of the reference range for TC and LDL-c than participants who did not take these drugs (both models, $p < 0.01$). On the contrary, TG showed more concentrations out of range in the high PF group than in the low PF group (both models, $p < 0.05$) and this significant association remained when lipid, monounsaturated fatty acids and were considered as cofounders (model 2, $p < 0.05$).

Table 29. Association between biomarkers and physical fitness.

Dependent variable: Glucose (mg/dL)					
Model 1		β	p-value	Model 2	
	Male	-1.962	0.002		Male
	Female	0.000			Female
	Age (yr)	-0.019	0.245		Age (yr)
	Low fitness	-0.433	0.282		Low fitness
	Medium fitness	-0.307	0.423		Medium fitness
	High fitness	0.000			High fitness
	Carbohydrates intake (g)	0.000	0.8969		Weight (kg)
	Mono&Disaccharides intake (g)	0.004	0.616		BMI (kg/m ²)
	Polysaccharides intake (g)	-0.002	0.756		FFM (kg)
	Yes Antidiabetic drug	4.366	0.000		TBW (Kg)
	No Antidiabetic drug	0.000			Yes Antidiabetic drug
					No Antidiabetic drug
Dependent variable: Uric acid (mg/dL)					
Model 1		β	p-value		
	Male	-2.249	0.000		
	Female	0.000			
	Age (yr)	-0.005	0.783		
	Low fitness	-0.135	0.663		
	Medium fitness	-0.420	0.145		
	High fitness	0.000			
	Protein intake (g)	0.006	0.261		

Dependent variable: Urea (mg/dL)					
Model 1		β	p-value		
	Male	-0.650	0.096		
	Female	0.000			
	Age (yr)	-0.038	0.165		
	Low fitness	-0.213	0.639		
	Medium fitness	0.059	0.897		
	High fitness	0.000			
	Protein intake (g)	-0.018	0.005		
Dependent variable: Creatinine (mg/dL)					
Model 1		β	p-value	Model 2	
	Male	1.431	0.000	Male	1.668
	Female	0.000		Female	0.000
	Age (yr)	0.017	0.402	Age (yr)	0.017
	Low fitness	-1.323	0.000	Weight (kg)	0.018
	Medium fitness	-0.696	0.062	Height (cm)	0.009
	High fitness	0.000		FFM (kg)	-0.035
	Protein intake (g)	0.007	0.288	Low fitness	-1.381
				Medium fitness	-0.727
				High fitness	0.000
				Protein intake (g)	0.008
Dependent variable: Total protein (g/L)					
Model 1		β	p-value	Model 2	
	Male	0.805	0.195	Male	0.607
	Female	0.000		Female	0.000
	Age (yr)	0.005	0.894	Age (yr)	0.038
	Low fitness	2.002	0.061	Low fitness	2.058
	Medium fitness	1.039	0.069	Medium fitness	1.048
	High fitness	0.000		High fitness	0.000
	Protein intake (g)	0.026	0.737	GOT (U/L)	-0.013
	Vegetal intake (g)	-0.050	0.539	GPT (U/L)	0.030
	Animal intake (g)	0.004	0.955	GGT (U/L)	-0.001
Dependent variable: Total cholesterol (mg/dL)					
Model 1		β	p-value	Model 2	
	Male	1.092	0.000	Male	1.110
	Female	0.000		Female	0.000
	Age (yr)	0.023	0.151	Age (yr)	0.025
	Low fitness	1.028	0.000	Low fitness	1.036
	Medium fitness	0.546	0.038	Medium fitness	0.600
	High fitness	0.000		High fitness	0.000

Model 1			Model 2		
Total cholesterol intake (mg)	β	p-value	Total cholesterol intake (mg)	β	p-value
No lipid-lowering drugs	-0.962	0.000	SFA intake (g)	-0.106	0.037
Yes lipid-lowering drugs	0.000		MUFA intake (g)	-0.109	0.033
			PUFA intake (g)	-0.148	0.025
			Lipid intake (g)	0.103	0.021
			No lipid-lowering drugs	-0.973	0.000
			Yes lipid-lowering drugs	0.000	

Dependent variable: HDL-cholesterol (mg/dL)					
Model 1	β	p-value	Model 2	β	p-value
Male	-1.829	0.000	Male	-1.964	0.000
Female	0.000		Female	0.000	
Age (yr)	0.002	0.910	Age (yr)	0.007	0.701
Low fitness	-0.901	0.007	Low fitness	-0.917	0.007
Medium fitness	-0.391	0.231	Medium fitness	-0.384	0.246
High fitness	0.000		High fitness	0.000	
Total cholesterol intake (mg)	-0.001	0.375	Total cholesterol intake (mg)	-0.001	0.172
No lipid-lowering drugs	-0.148	0.625	SFA intake (g)	0.091	0.119
Yes lipid-lowering drugs	0.000		MUFA intake (g)	0.068	0.225
			PUFA intake (g)	0.025	0.723
			Lipid intake (g)	-0.054	0.272
			No lipid-lowering drugs	-0.216	0.486
			Yes lipid-lowering drugs	0.000	

Dependent variable: LDL-cholesterol (mg/dL)					
Model 1	β	p-value	Model 2	β	p-value
Male	0.297	0.158	Male	0.240	0.262
Female	0.000		Female	0.000	
Age (yr)	0.026	0.132	Age (yr)	0.032	0.078
Low fitness	0.563	0.051	Low fitness	0.651	0.029
Medium fitness	0.488	0.065	Medium fitness	0.589	0.030
High fitness	0.000		High fitness	0.000	
Total cholesterol intake (mg)	0.237	0.180	Total cholesterol intake (mg)	-0.002	0.064
No lipid-lowering drugs	-1.090	0.001	SFA intake (g)	-0.079	0.165
Yes lipid-lowering drugs	0.000		MUFA intake (g)	-0.129	0.024
			PUFA intake (g)	-0.015	0.904
			Lipid intake (g)	0.009	0.061
			No lipid-lowering drugs	-1.130	0.001
			Yes lipid-lowering drugs	0.000	

Dependent variable: Triglycerides (mg/dL)							
Model 1		β	p-value	Model 2		β	p-value
	Male	-0.436	0.056		Male	-0.557	0.022
	Female	0.000			Female	0.000	
	Age (yr)	0.026	0.132		Age (yr)	0.006	0.745
	Low fitness	-0.720	0.013		Low fitness	-0.694	0.020
	Medium fitness	-0.387	0.170		Medium fitness	-0.312	0.274
	High fitness	0.000			High fitness	0.000	
Total cholesterol intake (mg)		0.000	0.803	Total cholesterol intake (mg)		-0.001	0.209
				SFA intake (g)		-0.116	0.053
	No lipid-lowering drugs	0.437	0.079	MUFA intake (g)		-0.166	0.007
	Yes lipid-lowering drugs	0.000		PUFA intake (g)		-0.123	0.104
				Lipid intake (g)		0.141	0.009
				No lipid-lowering drugs		0.423	0.095
				Yes lipid-lowering drugs		0.000	
Dependent variable: Haematocrit (%)							
Model 1		β	p-value	Model 2		β	p-value
	Male	-2.568	0.000		Male	-1.746	0.000
	Female	0.000			Female	0.000	
	Age (yr)	0.015	0.452		Age (yr)	0.015	0.567
	Low fitness	-0.429	0.249		Low fitness	-0.608	0.195
	Medium fitness	-0.480	0.134		Medium fitness	-0.497	0.211
	High fitness	0.000			High fitness	0.000	
					Red blood cells (10^6/mm3)	-5.559	0.000
Dependent variable: Hemoglobin (g/dL)							
Model 1		β	p-value	Model 2		β	p-value
	Male	-2.408	0.000		Male	-1.677	0.000
	Female	0.000			Female	0.000	
	Age (yr)	0.029	0.157		Age (yr)	0.028	0.221
	Low fitness	-0.575	0.100		Low fitness	-0.527	0.171
	Medium fitness	-0.433	0.192		Medium fitness	-0.434	0.232
	High fitness	0.000			High fitness	0.000	
					Red blood cells (10^6/mm3)	-3.137	0.000

Dependent variable: Iron (ug/dL)					
Model 1	β	p-value	Model 2	β	p-value
Male	0.405	0.307	Male	0.432	0.293
Female	0.000		Female	0.000	
Age (yr)	-0.010	0.714	Age (yr)	-0.012	0.660
Low fitness	-0.176	0.729	Low fitness	-0.326	0.528
Medium fitness	-0.069	0.874	Medium fitness	-0.204	0.646
High fitness	0.000		High fitness	0.000	
Iron intake (mg)	-0.008	0.163	B ₁₂ intake (mg)	0.010	0.714
			Folate intake (ug)	-0.003	0.033
			Iron intake (mg)	-0.008	0.152

Dependent variable: Ferritin (ng/dL)					
Model 1	β	p-value	Model 2	β	p-value
Male	-1.310	0.000	Male	-1.336	0.000
Female	0.000		Female	0.000	
Age (yr)	-0.001	0.951	Age (yr)	0.000	0.986
Low fitness	0.184	0.665	Low fitness	0.211	0.626
Medium fitness	0.067	0.843	Medium fitness	0.073	0.831
High fitness	0.000		High fitness	0.000	
Iron intake (mg)	0.032	0.114	B ₁₂ intake (mg)	0.010	0.641
			Folate intake (ug)	0.000	0.858
			Iron intake (mg)	0.029	0.149
			Lipid intake (g)	-0.053	0.284

Dependent variable: Total homocysteine (μ mol /dL)					
Model 1	β	p-value	Model 2	β	p-value
Male	-1.284	0.000	Male	-1.287	0.000
Female	0.000		Female	0.000	
Age (yr)	-0.046	0.014	Age (yr)	-0.047	0.014
Low fitness	-0.723	0.030	Low fitness	-0.716	0.033
Medium fitness	-0.334	0.283	Medium fitness	-0.329	0.285
High fitness	0.000		High fitness	0.000	
Vitamin B ₁₂ intake (ug)	0.035	0.080	Vitamin B ₁₂ intake (ug)	0.034	0.113
Yes Renin-angiotensin system drug	0.542	0.053	Folate intake (ug)		
No Renin-angiotensin system drug	0.000		Yes Renin-angiotensin system drug	0.541	0.053
Yes Beta blocker	0.352	0.445	No Renin-angiotensin system drug	0.000	
No Beta blocker	0.000		Yes Beta blocker	0.355	0.442
			No Beta blocker	0.000	

Dependent variable: Vitamin B ₁₂ (pg/mL)						
Model 1		β	p-value	Model 2		
	Male	-0.747	0.004		Male	-0.749
	Female	0.000			Female	0.000
	Age (yr)	-0.053	0.006		Age (yr)	-0.053
	Low fitness	-0.847	0.020		Low fitness	-0.757
	Medium fitness	-0.509	0.099		Medium fitness	-0.539
	High fitness	0.000			High fitness	0.000
	Vitamin B ₁₂ intake (ug)	0.019	0.253		Vitamin B ₁₂ intake (ug)	0.019
	Protein intake (g)	0.005	0.468		Folate intake (ug)	0.000
						0.265
						0.692
Dependent variable: Serum folate (ng/mL)						
Model 1		β	p-value	Model 2		
	Male	0.202	0.581		Male	0.130
	Female	0.000			Female	0.000
	Age (yr)	0.013	0.651		Age (yr)	0.015
	Low fitness	0.658	0.173		Low fitness	0.632
	Medium fitness	0.343	0.387		Medium fitness	0.372
	High fitness	0.000			High fitness	0.000
	Folate intake (ug)	-0.001	0.240		Folate intake (ug)	-0.001
					Vitamin B ₁₂ intake (ug)	0.030
						0.202
						0.368
Dependent variable: RBC folate (ng/mL)						
Model 1		β	p-value	Model 2		
	Male	0.784	0.034		Male	0.730
	Female	0.000			Female	0.000
	Age (yr)	0.018	0.479		Age (yr)	0.019
	Low fitness	0.051	0.904		Low fitness	0.017
	Medium fitness	0.086	0.826		Medium fitness	0.097
	High fitness	0.000			High fitness	0.000
	Folate intake (ug)	-0.001	0.404		Folate intake (ug)	-0.001
					Vitamin B ₁₂ intake (ug)	0.017
						0.358
						0.496
Dependent variable: Vitamin 25[OH]D (ng/mL)						
Model 1		β	p-value			
	Male	0.235	0.300			
	Female	0.000				
	Age (yr)	-0.016	0.351			
	Low fitness	0.221	0.442			
	Medium fitness	0.101	0.705			
	High fitness	0.000				
	Vitamin D intake (ug)	0.005	0.318			
	Calcium intake (mg)	0.000	0.688			

β; standardized coefficients. BMI; body mass index; FFM, fat free mass; TBW, total body water; SFA; saturated fatty acids; MUFA; mono-unsaturated fatty acids; PUFA; poly-unsaturated fatty acids; GOT; Glutamic oxalacetic transaminase, GPT; Glutamic-pyruvic transaminase, GGT; Gamma glutamyl transpeptidase; RBC folate, red blood cell folate. Level of significance $p < 0.05$.

4.6.5 Discussion

This study has shown that there were significant associations between PF and several biomarkers related to particular diseases. Our main results revealed that the participants from the low PF group showed lower vitamin B₁₂ and TG levels out of reference range, and higher values out of the reference range for tHcy, creatinine, TC, HDL-c and LDL-c. A high percentage of the sample population also presented vitamin 25[OH]D deficiency and high TC, glucose, uric acid, LDL-c and tHcy concentrations independently of the PF level. To the best of the authors' knowledge, there are not studies analyzing the relationship between abnormal/normal biomarkers values and PF groups in Spanish older adults through this holistic approach.

Regarding B-vitamins, the subclinical deficiency and deficiency of these vitamins are common in the older population (21,72) and both tend to decrease with age (200). A total of 51.8% of the studied population presented subclinical deficiency considering as cut-off point a concentration less than 352.3 pg/mL (21) (data are not shown). Severe vitamin B₁₂ deficiency causes an irreversible degeneration of the nervous system. Vitamin B₁₂ and folates are involved as coenzymes of numerous regulating enzymes (134).

The relation between vitamin B₁₂, sfolate, RBC folate and tHcy is an ongoing research area, especially if PF and PA are taken into account (258). Likewise, no single blood marker has been described which could evaluate effectively the ageing process (362). Surprisingly, participants from the low PF group were associated with lower vitamin B₁₂ levels out of the reference range. Additionally, participants included in the low PF group presented significant abnormal tHcy concentrations, but no association was found for sfolate and RBC folate and PF groups in our study. Joubert et al. indicated that participants from active and high PF groups presented significant higher levels of tHcy than the sedentary group measured by means of treadmill VO_{2 max} (174). Alomari et al. observed an inverse association between PA levels and tHcy. Also, they reported lower levels of tHcy in the participants with higher vs. lower PA even after adjusting for vitamin B₁₂ (11). In this sense, Murakami et al. did also not observe differences in

plasma tHcy controlling for age, sex, and folate intake according to PA groups. In our study, with advancing age (in males) vitamin B₁₂ deficiency and tHcy levels increased (241). Kuo et al. observed that high tHcy concentrations were contrariwise associated with cardiovascular fitness in female, but not in male (181). Likewise, Schoor et al. observed that female in the highest quartile of tHcy had a significantly lower physical performance than did those in the lowest quartile (355). A recent systematic review suggested that the positive result of increasing folic acid and vitamin B₁₂ supplementation and regular physical exercise to prevent hyperhomocysteinemia (142). Vitamin D deficiency has been previously published in all age stages (82,137,308) and is still an unsolved public health problem. In our population, a total of 67.9% presented vitamin D values below 29.99 ng/mL. Vitamin D is considered as a marker of the improvement of muscle condition, mainly in healthy elderly adults with lower PA measured by means of PA questionnaires (7). Al-Eisa et al. found that participants with low PA showed significantly lower 25[OH]D serum concentration than active participants (7). However, in our study there was no association between PF levels and vitamin 25[OH]D.

Another interesting finding in our study was that the participants from the low PF group presented major risk of TC and LDL-c abnormal concentrations and low HDL-c and TG concentrations than the high PF group. These results are in accordance with others authors (28,30,265). Furthermore, LDL-c showed significant relationship with PF groups when lipids intake was included into the statistical analysis but not with TC intake. These results confirm the relationship between lipids intake suggested by Siri-Tarino et al. (324), among others. Dvorak et al. observed that elderly participants with high levels of cardiorespiratory fitness, independently of their PA levels, presented lower concentrations of TG, TC, total HDL-c and low LDL-c (93).

In our study, uric acid and urea were analyzed but there were not significant differences between PF groups and, there was also no association between PF and abnormal values. These results could suggest that both blood biomarkers might increase after intensive exercise (57,258,347) but not as a consequence of performed PA during their lives. Low levels of total protein are associated with malnutrition (32). A significant relationship was obtained between total protein and PF groups ($p < 0.05$) and a major tendency for abnormal total protein values was observed between in the low and medium PF groups when compared to the high PF ($p > 0.05$). Furthermore, participant in the low PF group

showed high risk of presenting abnormal values for creatinine than participants in the high PF group ($p < 0.05$) in our study.

Hematocrit, hemoglobin and the red blood cell have been related to significant major chances of adverse health-status measured (i.e. multi-mobility, cognitive impairment, disability and mortality) (217). Nevertheless, in our study, we could not find these associations between hematocrit and hemoglobin and PF levels. This could be because similar concentrations were obtained in all PF groups. Low levels of hematocrit have been observed in trained persons mainly due to an increased plasma volume (205).

Several biomarkers are key in biological pathways and have been related to CVD, and PF can have an influence on these levels, therefore, the holistic approach analyzed in this study can contribute to a better understanding of this relationship. Dietary intake was considered in the analysis due to its role is becoming increasingly fundamental in decreasing the progression of chronic disease (126).

Limitations and strengths

The current study presents some limitations. Firstly, the cross-sectional design cannot determine the cause-effect relationship between PF and biomarkers. Secondly, all participants were volunteers; thus the outcomes are applicable only for the study group. Despite of the above-mentioned limitations, the PHYSMED project has several strengths. One of the strengths of our study was the use of clusters. Another strength is the inclusion of a battery of PF tests, a more objective and precise method than questionnaires. Also, clustering of PF produces an alternative approach to summarizing PF levels and it allows to have a realistic wide vision about behavioral patterns. Moreover, the strict standardization of the field work and the blood samples protocol among the cities which took part in the study was another strength.

4.6.6 Conclusions

In conclusion, the study shows new approaches about the relationship between biomarkers and PF. Blood markers of health were associated with PF status in older adults. Those presenting a better blood markers profile had higher PF. These markers could be used as a routine to assess PF levels for this kind of population and public health strategies should be implemented.

4.7 ARTICLE 7: AN INNOVATIVE CLASSIFICATION OF PHYSICAL FITNESS AND BLOOD MARKERS AND THEIR ASSOCIATION WITH SOCIOECONOMIC STATUS, BODY COMPOSITION AND HEALTH-RELATED STATUS AMONG SPANISH OLDER ADULTS

Aparicio-Ugarriza R, Diaz E, Barrios L, Bibiloni MM, Julibert A, Palacios G, Tur JA, González-Gross M. J Sci Med Sport (submitted). (JCR: 3.857).

4.7.1 Abstract

To create a novel classification of blood health markers and physical fitness (PF), and their relationship with body composition, quality of life and socioeconomic status as determinants of health-related behavior among Spanish older adults. **Methods:** A cross-sectional study was conducted in older adults (n=427; females 57%; 55-88 yr) from Madrid and Mallorca (Spain). PF was measured through a battery of validated tests. Fasted blood samples were analysed for haematology, routine biochemistry, homocysteine and vitamins B₁₂, vitamin D and folate. A total index was created combining blood markers and PF data. Body composition was measured by means of bioimpedance; quality of life (EuroQol-5D-3L) and socioeconomic status were measured by validated methods. Correspondence analysis and decision tree were used as statistical analysis. **Results:** There were significant associations between total index and education ($p<0.01$), current income ($p<0.001$), body mass index and waist-to-height ratio (WHtR) (both $p<0.001$). According to the results obtained by the decision tree model between the total index and several environmental variables, the main predictor outcome was education. Moreover, this decision tree described that participants with primary school education also presented higher WHtR, were more inactive and sedentary than the other participants (all statistically significant at the level of 95%). **Conclusions:** Socioeconomic status, abdominal obesity, PA and sedentarism should be considered when guidelines or interventions are described due to all of these variables have been identified as determinants of health-related behaviour taking into account the index of PF and blood biomarkers. More effective strategies could be identified understanding what can lead people to behave in this sense.

Key words: classification; health; physical fitness; physical activity; sedentarism; socioeconomic status.

4.7.2 *Introduction*

It is widely acknowledged that regular physical activity (PA) improves physical and mental functions as well as helps to prevent and reverse some effects of chronic diseases (223). The lack of PA is one of the foremost global risk factors for mortality (188,190). Additionally, sedentary behaviours (SB) are associated with adverse effects on health and are related to an increase of all cause-mortality (253,351).

Physical fitness (PF) has been shown to be strongest predictor of all-cause mortality and of diseases linked to particular mortality (43,143). It is also considered as a potent health marker (256,269).

Assessing biomarkers is a key to measure the impact of exercise on different systems, organs and tissues (49,258). Likewise, understanding associations between circulating biomarkers and PF across the adult lifespan could aid in better describing mechanistic pathways leading to disability (272). A combination of biomarkers could be useful to monitor PF; however, the concentrations of each biomarker depend on several factors such as sex, age, diet, training status or fatigue degree, among others (258).

Increasing life expectancy is leading to an increase in the older population; with large economic and social repercussions have also increased (122). Thus, good quality of life in older adults is an aim outcome and it transcends physical health (36). In this sense, the World Health Organization establishes that the socioeconomic environment, the physical environment, lifestyle and behaviours are determinants of good health. These factors differ depending on sex, education, income, ethnicity and disability (378). Furthermore, socioeconomic and cultural environment, lifestyle and health selected behaviour are specific to each nation (299).

The impact of socioeconomic status (SES) and PA on health in adulthood has been reported (212,262); providing evidence that older people in higher SES are more likely to keep high levels of PA (223). Additionally, older people socioeconomically disadvantaged have greater prevalence of chronic diseases and higher mortality rates (277). Regarding education status, higher education level usually correlates with healthier behaviour (9).

However, it is commonly analysed variables in an independent way, and there are few studies that have examined people as multifactorial approach. There are no studies that have created a combined and holistic classification of PF and blood markers among Spanish old adults, and the link with different social parameters and health markers. Hence, categorizing multiple domains of PF and blood marker patterns and charactering them on SES, PA and sedentarism, body composition and health-related status can provide information to define interventions and policies depending on the subgroups. Therefore, the aims of this study were: (1) to create a combined classification of PF and blood markers and, (2) to identify the relationship with body composition, perceived health status and socioeconomic status as determinants of the behavior in this sample.

4.7.3 Materials and Methods

Study design and sample

This paper is based on a cross-sectional multi-centre study aiming at identifying cardiovascular risk factors in active and sedentary elderly participants. Data collection took place from April 2013 to May 2014 in Madrid and Mallorca (Spain). Participants were recruited through health centres, sport federations, sport facilities and municipal clubs located at Madrid and Mallorca by snowball system. A sample of 433 participants, 184 male (43%) and 244 females (57%) aged 55 to 88 yr met the inclusion criteria. In the present study, 427 older adults (57% females) with valid data on blood parameters were included in the analyses. The exclusion criteria were age under 55 yr, being institutionalized, suffering from a physical or mental illness which would have limited their participation in the PF tests or their ability to respond to the questionnaires or drug intake for clinical research.

The study was performed following the ethical guidelines of the 1964 Declaration of Helsinki (revision of Seul 2008) and the protocol was approved by the Ethical Committee of the Technical University of Madrid. Written informed consent was obtained from each participant.

Physical examination

Height (m) was assessed to the nearest millimetre using a mobile stadiometer (SECA 213, Germany), with the participant's head in the Frankfurt plane. Body weight (kg) was measured using bioimpedance analysis (TANITA Corp, BC-418MA, Tokyo,

Japan) in standardized conditions. Participants were barefoot and in light clothing during anthropometric measurements. Body mass index (BMI) was calculated as the weight (kg) divided by the square of the height (m^2). Also, BMI was divided into three categories: normal weight ($<24.9 \text{ kg/m}^2$), overweight ($25.0\text{-}29.9 \text{ kg/m}^2$) and obese ($>30.0 \text{ kg/m}^2$). Waist circumference was measured as the smallest horizontal girth between the costal margins and the iliac crests at minimal respiration using a flexible, non-extensible plastic tape with 0.1 cm precision (Rosscraft), according to the methods of the ISAK society (210). Waist to height ratio (WHtR) was defined as waist circumference (cm) divided by height (cm). Additionally, a WHtR cut-off of 0.580 was considered following the criteria proposed by Bohr et al. (46).

Physical fitness

Prior to assess PF tests, blood pressure was measure using a digital monitor (Omron Healthcare Co., Ltd. Kyoto, Japan). Each participant completed a multi-component battery of PF tests validated in the elderly population (290) and validated reference ranges for Spanish older adults proposed by Pedrero-Chamizo et al. (269). Lower body strength was measured by the chair stand test (290), agility/dynamic balance was performed by the 8-foot up-and-go test (290), aerobic endurance was assessed by the 6-min walk test (290) and handgrip strength was measured with a handgrip dynamometer (Takei TKK 5401, Tokyo, Japan, range 5-100 kg, precision 0.1 kg) (361). The handgrip strength was assessed for both hands in a standing position. All tests were performed twice, except the 6-min walk test and the chair stand test, and the best score was retained.

The results of each PF tests were stratified by sex and age groups (distributed by five years periods without truncating the last group) following the criteria established by Pedrero-Chamizo et al. (269). Also, the result of each PF test was divided into quartiles considering the variables above mentioned (sex and age groups). The score for each test ranged from 0 (worst) to 3 (best) points. Thus, the maximum score was 12 points. Scores of PF tests were added together to create a cluster. After that, PF cluster analyses were performed and each cluster was divided into different levels. In order to classify our population taking into account analysis of clustering, we established three groups of PF: low, medium and high. The entire description is shown in Table 30.

Table 30. Description of the Physical Fitness Index score component for measuring physical fitness level among Spanish older adults.

Components	Description	Score*
Aerobic endurance	Number of meters that can be walked in 6 minutes around a 46 meters course. Participants were instructed to walk as far as possible for 6 min.	0 points= (p25] 1 point = (p25, p50] 2 points = (p50, p75] 3 points = (p75, +]
Agility/dynamic balance	Number of seconds required to rise from stated position, walk 2.45 meters, turn, and return to seated position on chair. Further instructions were to complete the test as quickly as possible.	0 points= (p75, +] 1 point = (p50, p75] 2 points = (p25, p50] 3 points = (p25]
Leg strength	The 30-s Chair Stand Test consists of the number of full stands in 30 seconds with arms folded across chest. Initially, subjects were seated on the chair with their back in an upright position. They were instructed to look straight forward and to rise after the “1, 2, 3, go” command at their own preferred speed with their arms folded across their chest.	0 points= (p25] 1 point = (p25, p50] 2 points = (p50, p75] 3 points = (p75, +]
Maximum handgrip strength	The participants squeeze gradually and continuously for at least 2 seconds, performing the test two times for each hand in stand position. The handgrip span was adjusted according to the hand size. The maximum score in kilograms for each hand was recorded.	0 points= (p25] 1 point = (p25, p50] 2 points = (p50, p75] 3 points = (p75, +]
Total score	Scores of physical fitness tests were added together and the maximum score was 12 points. After that, cluster analysis was performed and three levels of physical fitness were obtained.	Low fitness= 0-3 points (n=130)
		Medium fitness= 4-8 points (n=171)
		High fitness= 9-12 points (n=126)

p, percentile. *Score of each physical fitness tests was stratified by sex and age groups (distributed by five years periods without truncating the last group).

Physical activity and sedentarism

Our population was classified into 4 groups: inactive and low sedentary, inactive and high sedentary, active and high sedentary and active and low sedentary taking into account the classification proposed by Aparicio-Ugarriza et al. (22).

Blood samples

Blood samples were collected by standard venepuncture on vacuum Vacutainer® tubes at the biochemical laboratories of the High Sports Council (Madrid) and Hospital Son Espases & University of Balearic Islands (Mallorca) between 9:00am and 10:30 am after a 12h overnight fast. Blood samples with separation gel for serum or EDTA as anticoagulant were collected in the tubes. Gel for serum tubes were immediately placed on ice and after coagulum formation the sample was centrifuged at 3,000 rpm for 10 min. Haematocrit and hemoglobin analysis were performed within the first hour after extraction in EDTA tubes using an automated hematology analyser (ADVIA 120 Siemens Health Care Diagnostics, SA). Additionally, serum total cholesterol, HDL-cholesterol (HDL-c), LDL-cholesterol (LDL-c), triglycerides (TG), glucose, urea, uric acid, total protein, albumin, iron and ferritin were analysed using the Beckman AU400 analyser (Beckman AU400, Beckman Instruments, Ltd., Bucks, UK) by photometric methods. Creatinine was analysed by colorimetric method (Beckman AU400, Beckman Instruments, Ltd., Bucks, UK). These determinations were carried out in each site.

Vitamin B₁₂, serum folate, red blood cell folate (RBC folate), vitamin 25[OH]D and total homocysteine (tHcy) were centralized at the High Sports Council Laboratory in Madrid. Previously, the samples were stored at -80°C until assayed. RBC folate was measured using EDTA tubes. Therefore, serum samples from Palma de Mallorca were dry-ice shipped to Madrid and analysed together with those serum samples stored in Madrid at -80°C, by electrochemoluminescence method (Elecsys 2010, Roche Diagnostics, Switzerland), except vitamin 25[OH]D which was determined using E411 analyser (Roche Diagnostics, Switzerland).

Variables were categorized as being within reference range (coded as 1) or out of the reference range (coded as 0). Reference ranges which have been used were those established by each laboratory for adults obtained from population studies and published elsewhere (19). Scores of each biomarker were added together. The maximum score was 19 points. However, the maximum score of each participant was

considered using weighed average over their own maximum. After that, participants were grouped into 3 groups (low, medium and high level). The entire description and the cut-offs used are shown in Table 31.

Table 31. Description of the Biomarker Index score component for measuring health status among Spanish older adults.

Components	Description	Score
Glucose	Reference value: ≥ 110.0 mg/dL	≥ 110.0 mg/dL= 0 points < 110.0 mg/dL= 1 point
Urea	Reference value: ≥ 50.0 mg/dL	≥ 50.0 mg/dL= 0 points < 50.0 mg/dL= 1 point
Uric acid	Reference value: ≥ 5.9 mg/dL	≥ 5.9 mg/dL= 0 points < 5.9 mg/dL= 1 point
Creatinine	Reference value: ≥ 1.4 mg/dL males; ≥ 1.3 mg/dL females	≥ 1.4 mg/dL males; ≥ 1.3 mg/dL females= 0 points < 1.4 mg/dL males; < 1.3 mg/dL females= 1 point
Total protein	Reference value: ≤ 6.4 g/dL	≤ 6.4 g/dL= 0 points > 6.4 g/dL = 1 point
Albumin	Reference value: ≤ 3.0 g/dL	≤ 3.0 g/dL= 0 points > 3.0 g/dL= 1 point
Total cholesterol	Reference value: ≥ 200.0 mg/dL	≥ 200.0 mg/dL= 0 points < 200.0 mg/dL= 1 point
HDL-cholesterol	Reference value: ≤ 40.0 mg/dL males; ≤ 50.0 mg/dL females	≤ 40.0 mg/dL males; ≤ 50.0 mg/dL females= 0 points > 40.0 mg/dL males; > 50.0 mg/dL females= 1 point
LDL-cholesterol	Reference value: ≥ 150.0 mg/dL	≥ 150.0 mg/dL= 0 points < 150.0 mg/dL= 1 point
Triglycerides	Reference value: ≥ 150.0 mg/dL	≥ 150.0 mg/dL= 0 points < 150.0 mg/dL= 1 point
Haematocrit	Reference value: $\leq 35\%$	$\leq 35\%$ = 0 points $> 35\%$ = 1 point
Hemoglobin	Reference value: ≤ 15.5 g/dL	≤ 15.5 g/dL= 0 points > 15.5 g/dL= 1 point
Iron	Reference value: ≤ 60.0 μ g/dL	≤ 60.0 μ g/dL= 0 points > 60.0 μ g/dL = 1 point
Ferritin	Reference value: ≤ 30.0 μ g/dL	≤ 30.0 μ g/dL= 0 points > 30.0 μ g/dL= 1 point
Total homocysteine	Reference value: ≥ 15.0 μ mol/L	≥ 15.0 μ mol/L= 0 points < 15.0 μ mol/L= 1 point

Components	Description	Score
Vitamin B ₁₂	Reference value: ≤ 250.0 pg/mL	≤ 250.0 pg/mL = 0 points > 250.0 pg/mL = 1 point
Serum folate	Reference value: ≤ 6.8 nmol/L	≤ 6.8 nmol/L = 0 points > 6.8 nmol/L = 1 point
Red blood cell folate	Reference value: ≤ 212.0 ng/mL	≤ 212.0 ng/mL = 0 points > 212.0 ng/mL = 1 point
Vitamin D	Reference value: ≤ 20.0 ng/mL	≤ 20.0 ng/mL = 0 points > 20.0 ng/mL = 1 point
Total score	Scores of each biomarker were added together and the maximum score was 19 points. However, the maximum score of each participant was considered using weighed average over their own maximum. After that, participants were grouped in 3 groups by terciles.	Few of blood markers within reference range (n=171) [≤ 0.74]
		Mostly half of the blood markers within reference range (n=132) [0.75-0.84]
		Most of the blood markers within reference range (n=124) [≥ 0.84]

Lifestyle and Health-related quality of life (EuroQol, EQ-5D-3L) questionnaire

General EXERNET questionnaire was used including smoking habits. Participants were grouped into categories as follows: (i) educational level: primary school, secondary school and college-level education; (ii) current income: <600 €/month or low, 600-900€/month or medium and ≥ 900 €/month or high; (iii) smoker and non-smoker; (iv) civil status: single, married, divorced, widowed; (v) do you live alone: yes/ no.

Likewise, education and current income (education¤t income) were grouped together and the following categories were obtained: PS_CIlow, primary school and low current income (<600€/month); PS_CImed, primary school and current income (600-900€/month); PS_CIhigh, primary school and current income (>900€/month); SS_CIlow, secondary school and low current income (<600€/month); SS_CImed, secondary school and current income (600-900€/month); SS_CIhigh, secondary school and current income (>900€/month); UG_CIhigh, university graduate and current income (>900€/month).

In the present study, quality of life was assessed using the validated questionnaire EuroQol-5D-3L (29,101) which has been used in elderly population Olivares et al. (252). The EuroQool-5D-3L is composed by 5 different dimensions, namely mobility, self-care, daily activities, pain-discomfort and anxiety-depression. Participants were asked to indicate his/her health state in each dimension according to three different levels: (i) no problems, (ii) moderate problems, or (iii) severe problems.

Statistical analysis

Statistics were performed using statistical software SPSS (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp). Descriptive characteristics were summarized by calculating means and standard deviations (SD) unless otherwise stated. All variables were checked for normality of distribution using the Kolmogorov-Smirnov test. Regarding blood parameters, the urea, uric acid, total protein, albumin, TC, HDL-c, LDL-c, hemoglobin, haematocrit and 25[OH]D values showed normal distribution.

PF index and blood index were generated (tables 30 and 31) as stated above. In order to examine whether association existed between both indices, a Pearson χ^2 test was applied using Monte Carlo method; however, the relationship between both indices was

independent (data are not shown, $\chi^2=14.362$, $p>0.05$). Therefore, total index was combined both together.

The total index was divided into 9 categories: AA, most of the blood parameters within reference range and high PF level; AB, most of the blood parameters within reference range and medium PF level; AC, most of the blood parameters within reference range and low PF; BA, mostly half of the blood parameters were within reference range and high PF level; BB, mostly half of the blood parameters were within reference range and PF level; BC, mostly half of the blood parameters were within reference range and low PF level; CA, few of the blood parameters within reference range and high PF; CB, few of the blood parameters within reference range and medium PF level; CC, few of the blood parameters within reference range and PF level. Likewise, a total index was reduced to three categories (total index_3C): high (AA, AB, BA), medium (AC, BB, CA) and low (BC, CB, CC).

To describe the relationship between two categorical variables, cross-tabulations were applied between total index and education, current income, civil status, living alone or not, smoking, education¤t income, categorical BMI, WHtR, PA and sedentarism groups and health-related quality of life. After that, correspondence analysis was used in the variables that showed significance.

Finally, decision tree classification was applied in order to examine which variables are related to total index _3C and the method used was CRT. For a variable to remain in the decision tree model, a significance level of $p<0.05$ was required. Total index_3C was chosen in the decision tree due to presented higher number of participants in each category than total index. In all the analyses, $p<0.05$ were considered statistically significant.

4.7.4 Results

Figure 24 shows the number of participants included in the PF index and blood index.

n, number of participants.

Figure 24. Number of participants in each group of physical fitness index and blood index.

Table 32 displays the number of participants in each category of the total index combining blood and PF indices.

Table 32. Number of participants in each category.

Index	n	%
AA	38	8.9
AB	41	9.6
AC	47	11.0
BA	49	11.5
BB	51	11.9
BC	71	16.6
CA	37	8.7
CB	40	9.4
CC	53	12.4

n, number of participants; %, percentage of participants in each group; AA, most of the blood parameters within reference range and high PF level; AB,

most of the blood parameters within reference range and medium PF level; AC, most of the blood parameters within reference range and low PF; BA, mostly half of the blood parameters were within reference range and high PF level; BB, mostly half of the blood parameters were within reference range and PF level; BC, mostly half of the blood parameters were within reference range and low PF level; CA, few of the blood parameters within reference range and high PF; CB, few of the blood parameters within reference range and medium PF level; CC, few of the blood parameters within reference range and PF level.

Table 33 shows total index and socioeconomic variables, BMI, WHtR and health-related questions. A significant correlation was found between total index and education ($p<0.01$), current income ($p<0.001$), education¤t income ($p<0.001$), BMI ($p<0.001$). A lineal by lineal association was observed between total index with WHtR ($p<0.001$), PA and sedentarism ($p<0.001$), pain or discomfort and anxiety or depression ($p<0.05$). The variables that did not show significant associations did not remain in the application of correspondence analysis and decision tree analysis.

Table 33. Total index and socioeconomic variables, body composition and health-related quality of life.

	Total index	
	χ^2 Pearson	p-value
Education	34.774	0.004
Current income	46.671	<0.001
Civil status	18.769	0.769
Do you live alone?	6.249	0.619
Do you smoke?	10.989	0.195
Education¤t income	83.932	<0.001
Categorical BMI	44.348	<0.001
WHtR†	24.642	<0.001
PA and sedentarism groups†	13.468	<0.001
Mobility	11.917	0.155
Personal care	5.098	0.890
Daily activities	8.872	0.373
Pain or discomfort†	4.712	0.040
Anxiety or depression†	5.479	0.020

BMI, body mass index, WHtR, waist to height ratio. †For these variables, lineal by lineal association was considered. Level of significance $p<0.05$.

Figures 25-31 depict the relationship between total index and different variables (education, current income, PA and sedentarism cluster, categorical BMI, education & current income, pain or discomfort and anxiety or depression). There were significant associations between total index with education and current income ($p<0.01$ and $p<0.001$, respectively). Also, significant relationships were found between categorical

BMI (normal weight, overweight and obese), education¤t income and WHtR (data are not shown due to this variable presented one dimension) with total index (all $p<0.001$).

AA, most of the blood parameters within reference range and high PF level; AB, most of the blood parameters within reference range and medium PF level; AC, most of the blood parameters within reference range and low PF; BA, mostly half of the blood parameters were within reference range and high PF level; BB, mostly half of the blood parameters were within reference range and PF level; BC, mostly half of the blood parameters were within reference range and low PF level; CA, few of the blood parameters within reference range and high PF; CB, few of the blood parameters within reference range and medium PF level; CC, few of the blood parameters within reference range and PF level. Correspondence analysis was carried out. Level of significance $p<0.05$.

Figure 25. Association between total index (fitness&blood) with education.

AA, most of the blood parameters within reference range and high PF level; AB, most of the blood parameters within reference range and medium PF level; AC, most of the blood parameters within reference range and low PF; BA, mostly half of the blood parameters were within reference range and high PF level; BB, mostly half of the blood parameters were within reference range and PF level; BC, mostly half of the blood parameters were within reference range and low PF level; CA, few of the blood parameters within reference range and high PF; CB, few of the blood parameters within reference range and medium PF level; CC, few of the blood parameters within reference range and PF level. Correspondence analysis was carried out. Level of significance $p < 0.05$.

Figure 26. Association between total index (fitness&blood) with current income.

AA, most of the blood parameters within reference range and high PF level; AB, most of the blood parameters within reference range and medium PF level; AC, most of the blood parameters within reference range and low PF; BA, mostly half of the blood parameters were within reference range and high PF level; BB, mostly half of the blood parameters were within reference range and PF level; BC, mostly half of the blood parameters were within reference range and low PF level; CA, few of the blood parameters within reference range and high PF; CB, few of the blood parameters within reference range and medium PF level; CC, few of the blood parameters within reference range and PF level. Correspondence analysis was carried out. Level of significance $p < 0.05$.

Figure 27. Association between total index (fitness&blood) with physical activity and sedentarism cluster.

AA, most of the blood parameters within reference range and high PF level; AB, most of the blood parameters within reference range and medium PF level; AC, most of the blood parameters within reference range and low PF; BA, mostly half of the blood parameters were within reference range and high PF level; BB, mostly half of the blood parameters were within reference range and PF level; BC, mostly half of the blood parameters were within reference range and low PF level; CA, few of the blood parameters within reference range and high PF; CB, few of the blood parameters within reference range and medium PF level; CC, few of the blood parameters within reference range and PF level. Correspondence analysis was carried out. Level of significance $p < 0.05$.

Figure 28. Association between total index (fitness&blood) with categorical body mass index.

AA, most of the blood parameters within reference range and high PF level; AB, most of the blood parameters within reference range and medium PF level; AC, most of the blood parameters within reference range and low PF; BA, mostly half of the blood parameters were within reference range and high PF level; BB, mostly half of the blood parameters were within reference range and PF level; BC, mostly half of the blood parameters were within reference range and low PF level; CA, few of the blood parameters within reference range and high PF; CB, few of the blood parameters within reference range and medium PF level; CC, few of the blood parameters within reference range and PF level. PS_CIlow, primary school and low current income (<600€/month); PS_CImed, primary school and current income (600-900€/month); PS_CIhigh, primary school and current income (>900€/month); SS_CIlow, secondary school and low current income (<600€/month); SS_CImed, secondary school and current income (600-900€/month); SS_CIhigh, secondary school and current income (>900€/month); UG_CIhigh, university graduate and current income (>900€/month). Correspondence analysis was carried out. Level of significance $p < 0.05$.

Figure 29. Association between total index (fitness&blood) with education¤t income.

AA, most of the blood parameters within reference range and high PF level; AB, most of the blood parameters within reference range and medium PF level; AC, most of the blood parameters within reference range and low PF; BA, mostly half of the blood parameters were within reference range and high PF level; BB, mostly half of the blood parameters were within reference range and PF level; BC, mostly half of the blood parameters were within reference range and low PF level; CA, few of the blood parameters within reference range and high PF; CB, few of the blood parameters within reference range and medium PF level; CC, few of the blood parameters within reference range and PF level. Correspondence analysis was carried out. Level of significance $p < 0.05$.

Figure 30. Association between total index (fitness&blood) with pain or discomfort.

AA, most of the blood parameters within reference range and high PF level; AB, most of the blood parameters within reference range and medium PF level; AC, most of the blood parameters within reference range and low PF; BA, mostly half of the blood parameters were within reference range and high PF level; BB, mostly half of the blood parameters were within reference range and PF level; BC, mostly half of the blood parameters were within reference range and low PF level; CA, few of the blood parameters within reference range and high PF; CB, few of the blood parameters within reference range and medium PF level; CC, few of the blood parameters within reference range and PF level. Correspondence analysis was carried out. Level of significance $p < 0.05$.

Figure 31. Association between total index (fitness&blood) with anxiety or depression.

Figure 32 displays the number of participants in the reduced index including each category of the total index. The decision tree showed a reasonable overall fit and explained 100%.

n, number of participants; %, percentage of participants in each group; AA, most of the blood parameters within reference range and high PF level; AB, most of the blood parameters within reference range and medium PF level; AC, most of the blood parameters within reference range and low PF; BA, mostly half of the blood parameters were within reference range and high PF level; BB, mostly half of the blood parameters were within reference range and PF level; BC, mostly half of the blood parameters were within reference range and low PF level; CA, few of the blood parameters within reference range and high PF; CB, few of the blood parameters within reference range and medium PF level; CC, few of the blood parameters within reference range and PF level.

Figure 32. Number of participants in the total index (three categories) including each category of total index (nine categories) by decision tree.

Figure 33 shows decision tree classification with total index_3C and all variables. The most discriminant classification found is shown in the tree, being all partitions statistically significant at 95%. The main predictor variable for the total index_3C was education which presented two groups (primary school and secondary school or more). In the left branch (primary school), the following predictor variable was PA and sedentarism groups (improvement=0.016) from which 60.9% of participants were in the active and low sedentary group and also, in the low WHtR. In the right branch (secondary school or more), the following predictor variable was WHtR (improvement=0.075), and 15.9% of participants with high WHtR, a total of 64.7% of participants were in the low PF and blood total index_3C.

WHtR, waist to height ratio; PA/SB, physical activity and sedentary behaviour groups. n, number of participants; %, percentage of participants in each group; AA, most of the blood parameters within reference range and high PF level; AB, most of the blood parameters within reference range and medium PF level; AC, most of the blood parameters within reference range and low PF; BA, mostly half of the blood parameters were within reference range and high PF level; BB, mostly half of the blood parameters were within reference range and PF level; BC, mostly half of the blood parameters were within reference range and low PF level; CA, few of the blood parameters within reference range and high PF; CB, few of the blood parameters within reference range and medium PF level; CC, few of the blood parameters within reference range and PF level. Statistically significances at level 95%.

Figure 33. Decision tree with total index (three categories) and education, physical activity and sedentarism groups, waist to height ratio and age.

4.7.5 Discussion

The goal of this study was to create a novel index combining several biological markers of health as PF and blood markers and to examine the associations among the participant's environment. These findings afforded the opportunity to evaluate the relationships utilizing correspondence and decision tree analyses. Our study showed that PF and blood indexes are independent; nevertheless, significant associations were found between total index and SES, BMI and WHtR. Also, the decision tree model between total index and total index_3C classified a reasonable total fit and explicated 100% and the other decision tree showed that all partitions presented statistical significances at 95%. Through the decision tree, participants in the low PF and blood level of the total index_3C presented higher possibility of being associated with high WHtR, inactive PA groups and active and high sedentarism group.

The current study's findings are consistent with several studies which found that education was associated to health (9,223,277). Nevertheless, a recent systematic review (279) found that higher education levels were not significantly associated with moderate to vigorous PA (MVPA) and income showed a lack of effect on MVPA, especially in experimental and prospective studies (356). Likewise, civil status, smoking and living alone did not show associations with total index. In this line, Prince et al. did not observe correlations between marital status and MVPA (279). On the contrary, Waite reported that being married or living in a couple is a protective factor on overall health (363). Manfredini et al. showed that being married was related to lower risk factors and better health status, even in consideration of several confounding effects (207). Married males presented higher median levels of exercise participation and married females performed higher level of total PA (273).

It is important to bear in mind that to maintain an active lifestyle and intellectual well-being is connected with a high quality of life during old age (325). In our study, we observed a significant correlation between anxiety or depression and pain or discomfort with low levels of the total index. This finding supports a previous report that found that higher levels of anxiety were associated with lower MVPA levels (196). On contrary, Allan et al. did not observe a significant relationship between anxiety and PA level (9). Arai et al. also found that socially inactive older adults were frequently depressed in spite of similar PA levels (23). In our study, there were no associations between total index with mobility, personal care and daily activities measured by means of the

EuroQol questionnaire. This may be explained because the age of participants. In general, our participants are in the middle-ages and older adults. In fact, the decision tree did not show differences between age groups. Participants did not perceive problems in mobility, personal care and performing daily activities. All in all, PA seems to act as partial mediator of the relationship between SES and health, and other factors such as chronic stress may be considered (348). This study's finding suggests that category CB (with few of the blood parameters within reference range and medium PF level) from the total index and PF level seems to be associated with moderate anxiety or depression. Nonetheless, both factors were not shown as significant variables in the decision tree.

In our study, a significant correlation was found between total index and PA and SB groups. Concretely, participants in the high total index_3C level were associated with the active and low SB group, but participants in both inactive groups and active and high SB group were related to a low total index level. Although there are no studies analysing the relationship between PF and blood markers together and SES, Omoru et al. found that participants with low SES performed occupational PA with higher energy expenditure. Meanwhile, those with high SES performed less occupational PA with less energy expenditure (253). Other authors observed that low SES participants with high occupational PA are also willing to employ time in active-transportation or leisure-time PA (111,343). In this sense, considering the results from the decision tree, participants in the low PF and blood level with primary school were involved in less time doing PA and more SB than the other groups.

Regarding blood markers of health, these were associated with PF status in older adults (19). Those presenting a better blood markers profile had higher PF; however, SES was not taken into account in that study. Brown et al. reported that the biological mechanisms explaining the relation between PA and cardiovascular disease mediated by SES are not well understood (54). Likewise, they found that SES intermediates the association between lipid markers and PA (54). Participants in the low total index showed association with lower education and current income level. The decision tree determined that education was the main variable and in the right branch (secondary school or more), the following variable was WHtR, and 15.9% of participants with high WHtR, a total of 64.7% of participants were in the low PF and blood total index_3C.

Recently, WHtR has been proposed as early health risk marker (27) and superior to BMI in detecting cardiometabolic risks in both sexes (27,227). WHtR is an appropriate anthropometric index according to age, sex, race and genetic independently of the changes performed by PF level and body weight (118). Likewise, abdominal obesity is related to metabolic abnormalities, systemic inflammation, insulin resistance, impaired glucose, among others (46,227). In this study, an important association was observed between total index_3C and WHtR with participants in the low level of the total index showing higher WHtR. Also, participants in the active and sedentary group also had lower levels of WHtR than the other groups. Lee et al. also observed that active participants were related to lower prevalence of WHtR and BMI (191).

It is likely that different interventions are needed to successfully increase PF level and consequently, PA levels and to improve blood marker concentrations in older adults (188). Therefore, to classify people taking into account their overall environment is vital in order to reduce SB, overweight, obesity and other chronic diseases and as a result improve their independence and quality of life.

This study presents some limitations that are important to note. First, the primary limitation of the cross-sectional study design is that because the exposure and outcome are simultaneously assessed, there is generally no evidence of a temporal relationship between exposure and outcome. Second, the use of self-reported questionnaires which could imply reporting bias and also, the studies population consisted of participants who joined voluntarily. Besides these limitations, our study presents several strengths such as a novel and holistic classification created combining PF and blood biological markers of health. Moreover, this is the first report concerning PF and blood index and their relationship with SES, body composition, health-related and PA and sedentarism in Spanish older adults.

4.7.6 Conclusions

PF and blood indices must be considered as independent factors. The combination of both indices allows addressing the current problematic as multifactorial and holistic approach, and also allows to discriminate which variables are most significant. The total index_3C showed partitions statistically significant for education, PA and SB groups, WHtR and age. Furthermore, BMI, current income, anxiety and pain correlated significantly with total index; thus, these variables should be measured. The holistic

information provided in this study should be taken into account to identify more effective strategies, policies and targeted interventions for different groups of people.

5 INTEGRATIVE DISCUSSION

This thesis aims at analysing the associations between health markers with physical activity (PA), sedentary behaviour, hydration habits, nutritional status, oxidative stress parameters, socioeconomic status and quality of life in Spanish older adults. Furthermore, classifying participants according to subjective and objective methods and its relationship with all abovementioned variables. Results contribute to enable more operative interventions from a clinical and public health point of view. This part will summarize the results of the different articles and discuss in common with those described in the literature.

The PHYSMED Study is one of the few studies that provide data on main health factors in Spanish older adults; therefore, the current thesis has several strengths: (i) it is based on the validated EXERNET methodology but broader as it also includes blood biomarkers, dietary and hydration status. Additionally, nutritional status assessment is based on the PREDIMED study. (ii) the sampling procedure and the strict standardization of the field work among the cities involved in the study avoided to a great extent the kind of confounding bias due to inconsistent protocols and different laboratory methods which makes comparing results from isolated studies difficult. Analysis of sex, age, body composition, dietary intake, hydration status, PA and physical fitness (PF) were assessed simultaneously and centralized in a common database. These factors are commonly analysed independently and this approach might bring on misleading results. Up to now, several studies only assess PA but not fitness, PA and PF but not sedentary behaviour, PF but not blood markers and this is a further strength of our work. Likewise, no single biomarker from functional to blood will offer a valid measure of healthy aging; thus, measures of physical capability and blood biomarkers are a key goal of this thesis.

5.1 Different classifications from a clinical and public health point of view

The health of older adults is a major concern in our society. To achieve a healthy ageing and wellbeing are current goals in our society (186). PA recommendations proposed by international organisms (275,380) are well-known; however, it is a difficult task to establish effective policies and interventions classifying people according to these guidelines. One of the main scopes of the current thesis has been to try to clarify the difficulty to classify people analysing variables as a whole in order to obtain more reliable data.

In a previous study, Marin (211) compared our participant's inclusion criteria to different classifications of PA and sedentary behaviour proposed in the literature (77,275,285,331,380). Marin observed a lack of consensus in determining PA and sedentary behaviour among the identified classifications. The same subjects were classified in a range from very sedentary to very active depending on the classification used (211). This inconsistency leads both subjects and policy makers a) not to know how active or inactive they really are; b) which recommendation is suitable for them to maintain and/or improve their health. The increase in obesity and other non-communicable diseases in the last years (199), in spite of all efforts from public organisms, lead us to think that misclassification is one of the causes.

In this thesis, four different classifications have been proposed: (i) according to PA and sedentary behaviour (Article 2), (ii) according to PF groups (Articles 3-6), (iii) considering reference ranges from blood markers (Articles 6-7), (iv) combining (PF & blood markers) in a total index (Article 7). Firstly, a classification combining PA and sedentary behaviour was created. In spite of the limitations (372), validated questionnaires were used to evaluate PA and sedentary behaviour in our population (96,97,202). This is an accepted methodology in epidemiological studies (25). Furthermore, the inclusion of sedentary behaviour was a novel and key strength because these variables usually have been analysed in an independent way. Another strength was that two questionnaires were added together for cluster analyses in order to reduce bias. Both questionnaires showed significant correlation between them. Cluster analysis is a relative new technique in classifying participants with an important internal validity (hierarchical and non-hierarchical procedure) (253,300). The idea to combine PA and sedentary behaviour is supported because both variables are two different constructs (too much time spend sitting is different from too less time doing PA) (253).

The proposed classification is valid in males as it classifies participants according to their PA and sedentary behaviour and its relationship with PF tests, except for handgrip strength. Additionally, it can be used as a good estimator of overall health (77,260,366) when time and economic requirements do not allow to perform more objectives methods. However, this classification cannot be considered definitive (119), since a proper individual evaluation would require additional testing, especially for females and handgrip strength test.

Secondly, we decided to create an objective classification by means of PF markers, because the measurement of PF is an essential marker of current and future health (362). In the current thesis, PF is considered as a whole considering lower body strength, handgrip strength, agility and dynamic balance and aerobic capacity. Likewise, PF results were divided into quartiles based on sex and age, as proposed in other previous studies (55,235,313). The final PF score was split into three groups: low, medium and high as presented in articles 3-7 of this thesis. However, static balance, upper body strength, flexibility and 30-m walk test measured by means of the Rikli & Jones battery (290) were not considered in our PF classification. Static balance test does not allow discriminating those participants who had better performance. Participants presented several problems with dumbbells and proper execution technique for the upper body strength test (especially females). Subjects with injuries and/or several diseases (i.e. osteoarthritis, low back pain) had difficulties in performing the flexibility tests. We also observed difficulties in understanding the contradictory message of “walk as fast as possible without running” for the 30-m walk (speed test). Nevertheless, the inclusion of the results from the agility test guaranteed the inclusion of the speed capacity in the score. From a clinical and public health point of view, the offered PF classification is adequate to evaluate physical fitness level and international organism could use this classification to establish policies, strategies and interventions. Nonetheless, more studies are necessary in order to improve the methodology to examine upper body strength, flexibility and speed in this kind of population.

Thirdly, another classification was proposed to categorise participants according to their blood concentrations. Biomarkers offer objective physical or biologic measures (95,298); however, the identification of biomarkers is further complicated due to the diversity of biological living situations and lifestyle activities (362). Biomarkers studies are important to further understand the link between PF, PA and sedentary behaviour

with health outcomes (371). According to the results and in line with the literature, the correct interpretation of biomarkers requires well-defined reference values for the respective marker (95). As we observed in Article 1, there is still a lack of consensus about the normal reference range, especially in older adults. Although serum concentrations below the reference range are not necessarily related to deficiency (95), maintaining concentrations out of the reference range for a long time could have negative effects on health (402). In this sense, Sebastiani et al. supported that dysregulation of a single biomarker can change with patterns of other biomarkers, and age-related changes of individual biomarkers alone do not essentially indicate disease or functional decline (317). Besides, the effect of acute or regular exercise could be explaining the differences observed in Article 6. This classification responds to both a clinical and public health approach so that it can be used to evaluate people and to propose new public health interventions.

In the fourth place, there is a diversity of biological responses to living situations and lifestyles (362). We decided to classify participants according to PF and blood markers as these parameter are able to evaluate such responses (total index) (Article 7). Moving from a simple approach based on one biomarker at a time to a systems analysis approach that simultaneously mixes multiple biological markers provides a chance for a more holistic approach (385). Nevertheless, both PF and blood markers must be evaluated separately as one does not describe the other; therefore, a combined classification of PF and blood markers (total index) was created. To the best of our knowledge, this is the first investigation including PF and blood markers in an integrative classification. Total index was compared to body composition and several environmental factors, among others. Socioeconomic status includes education, income and occupational level and our results showed that the main predictor in the model was education. It has been previously reported that university education is associated with high PA and PF levels (9,223,277); nonetheless, others authors have found contrary results (111,253,279). According to health-related quality of life, we observed a negative association between a high total index score and anxiety or depression. It is also important to note that according to total index, participants with primary school education, but categorised in the active and low sedentary group, were associated with less abdominal obesity (waist-to height ratio).

There is a necessity of considering the life context of people in regards to its effect on health impact and behaviour determinants. This holistic approach will contribute to a better comprehension of which current markers of health will be more useful to give advice to Spanish older people with higher risk of disease. Depending on the scope of the intervention, these 4 classifications can be used from a clinical and public health point of view.

5.2 The associations between the different classifications with nutritional status, hydration habits, oxidative stress and environmental factors

Through the proposed classifications, dietary and fluid intake, oxidative stress parameters, body composition, socioeconomic status and quality of life have been analysed.

Participants in the active groups presented higher PF results than participants in the inactive groups. Indeed, males in the active and low sedentary group showed significant associations with all PF tests, except for handgrip strength (Article 2). Handgrip strength is a quick and easy indicator of muscle status (45) and it is considered an essential part of the evaluation of frailty and physical functioning in clinical settings (18,67,358). One of the reasons could be that our participants are younger than in other studies (and they are more fit than other populations). Additionally, cognitive status has been identified as the main influencing variable on handgrip strength (269) and our subjects had no cognitive impairment as they were able of fulfilling all tasks within the study. Furthermore, differences between sexes could be due to the menopause period that could increase inflammatory markers (18,56,110,166) and these could have an effect on their PF level. In line with inflammatory markers, different results were found between lipid peroxidation and antioxidant enzyme activity with several lifestyles in both sexes (Article 5). Females displayed a negative relationship between malondialdehyde (MDA) with PF, whereas high levels of PF were associated with high superoxide dismutase (SOD) activity in males. Several studies are in agreement with our results (192,193,261). Fraile-Bermúdez et al. observed that moderate-to vigorous PA was associated with a decrease in the total antioxidant status in elderly females (116). Sureda et al. analysed the trace elements content in the toenails in the regarding PHYSMED Study. There were no differences between active and inactive males and

trace elements. Conversely, active females showed higher calcium, chromium, iron, cobalt, and zinc and lower mercury contents than inactive peers (341). Comparing both sexes between them, inactive females showed lower calcium and cobalt levels than inactive males. Active females had higher levels of chromium than active males (341). Participants in the active groups had higher fluid intake than the participants in the inactive groups (Article 3). However, there was not a significant association between oxidative stress parameters and PA and sedentary behaviour classification in males and females, except for catalase in females. Likewise, participants in the active and low sedentary group consumed greater amounts of water, followed by juice, milk, coffee, sport drink, beer, wine and distilled drinks than the other groups. Nissensohn et al. found that the main fluid consumed was water, followed by milk in both sexes (249). Older adults (65-75 yr) of both sexes consumed higher amounts of hot drinks and alcohol and fewer of soft drinks and juices (249). Neither PA nor PF were considered by Nissensohn et al. (249) and comparison between studies is difficult. Both PA and PF must be taken into account as individual factors that can have effect over the total fluid intake. Regarding PF, we did not observe significant differences between PF groups in relationship to water and other beverages intake, except for distilled alcoholic drinks.

Another point of this thesis is to consider macro and micronutrient intake according to PF (Article 4). Dietary mediators play an essential role in the pathogenesis of cardiovascular diseases (280); therefore, understanding the effect of diet on metabolic pathways and homeostatic control (152) and their relationship with PF could help to elucidate public policies and interventions. Nevertheless, there are not studies analysing the association between PF and dietary intake in the Spanish population. Our results showed that PF seems to have an effect on dietary intake. A higher intake of macronutrients was observed in the high PF group when compared to the low PF group, concretely in males. Total mean daily energy intake in the PHSYMED Study was higher than in the ANIBES Study (304) but comparisons between studies are problematic because we examined dietary intake considering PF level. Higher associations were found between micronutrients and PF compared to macronutrient intake. Our results are similar to those obtained by Cao et al. (60). Indeed, participants in the high PF group presented higher micronutrient intake compared to the other PF groups. Participants in the low PF group showed higher inadequate intake for potassium, vitamins (A, B₃, B₅, B₆, B₉, C, D, and E) compared to the high PF group. In

line with our results, several authors found that active and fit people had higher energy intake (58,60). Hardly all micronutrients are required by the cells and tissues and may undergo a specific metabolism within the cells (37). Micronutrient deficiencies contribute to several age-related disorders and older adults are a risk group for micronutrient deficiency (152).

Independently of PF level, more than 85% of the participants showed inadequate intake for potassium, vitamin D and vitamin E below estimated average requirements (EAR). It is to note that 8.3% of the population showed inadequate intake of 11 micronutrients; consequently, there is an urgent need to ensure adequate micronutrient intake. Some studies are in accordance with our results (60,112,297). Individuals with a micronutrient intake below EAR could develop clinical signs and symptoms of deficiency depending on the duration of the intake below EAR and individual needs (37). A low intake of micronutrients could have long-term health effects and might contribute to the risk of chronic diseases (37). Additionally, changes in blood concentrations of such nutrients only appear in progressed/severe deficiency states (95). In this sense, we observed a positive relationship of MDA with vitamin C, glutathione peroxidase with vitamin A, myeloperoxidase with vitamin E, and SOD with selenium in males. In females, catalase (CAT) showed a negative association with vitamin E (Article 5). Furthermore, we found that CAT Lmf was related to greater intake of fruits per day in females. The results obtained are in agreement with other authors (148,192,204); however, a negative association was found between vitamin E intake and CAT Plm for females in our study. This may be due to that 96% of the females showed vitamin E intake below EAR; nonetheless, less than 50% of the participants showed intake below EAR for selenium, vitamin A and vitamin C in the current thesis.

Several nutritional and blood markers were evaluated in the current thesis (Article 6) and our results reveal that a high percentage of participants presented values out of reference range for vitamin 25[OH]D (68%), total cholesterol (TC) (59%), glucose (38%), uric acid (32%), low density lipoprotein cholesterol (LDL-c) (28%) and total homocysteine (tHcy) (24%), among others. Reliable information about nutritional status is needed to identify potential critical nutrients and individuals at risk of deficiency (95). Indeed, 52% of the population presented subclinical deficiency of vitamin B₁₂ considering one of the most common cut-off points found in Article 1. Nevertheless, vitamin B₁₂ and folate intake did not show significant association with vitamin B₁₂ and

its co-markers as blood marker (Articles 4 and 6). Unexpectedly and adding PF into this relationship, participants in the low PF group showed less vitamin B₁₂ and triglycerides values out of the reference range than in the high PF group but higher intake of vitamin B₁₂ than the other groups. Another main finding of this thesis is that more abnormal values were observed for tHcy, creatinine, TC, high density lipoprotein cholesterol and LDL-c in the low PF level. All lipid markers except TG showed greater abnormal concentration in the low PF group than in the high PF group. Some authors are in concordance with our results (28,30,265). It is remarkably that lipid intake is associated with serum lipid markers and PF level. Nonetheless, other markers such as folates, iron, albumin, total protein or urea did not reveal any relationship with PF due to participants are within reference values or may be other cut-off should be considered for older adults.

Limitations

Each article includes a specific section about limitations. Nevertheless, it is important to summarize the principal limitations of the thesis. The study sample consisted of volunteers, thus it is not possible to generalize the results to individuals who differ from the characteristics of our study sample. Since participation was voluntary, older adults with a low fitness level or with some health problems might have been less likely to participate in the study. Additionally, the cross-sectional design cannot determine the cause-effect relationships between variables. Finally, the sample size is not a representative sample of the Spanish older adults.

6 CONCLUSIONS

6.1 General Conclusion

The obtained data in the current thesis are novel which aim analysing the associations between physical fitness and several environmental and biochemical variables in Spanish older adults. The holistic approaches established have allowed getting deeper into these relationships. Physical fitness level showed association with total nutrient intake, especially vitamins and minerals, whereas hydration habits seem to be affected by physical activity level and sedentary behaviour. Physical activity and sedentary behaviour classification showed a significant relationship with physical fitness tests in males. Participants in the high physical fitness level showed a better health marker profile. It is important to note that sex was identified as the main factor in the association with oxidative stress parameters. Additionally, body composition and socioeconomic status should be evaluated as they have been identified as significant in the proposed total index.

Article 1

- A discrepancy in the literature related to vitamin B₁₂ and its co-markers cut-off points has been identified. Age, sex, fortified food consumption and analytical methods should be considered to establish cut-offs for vitamin B₁₂ and its co-markers in the clinical setting, especially in older adults.

Article 2

- The proposed physical activity and sedentary behaviour classification is valid as it discriminates according to the physical fitness level in males; however, the proposed classification does not differentiate between physical fitness levels in females.
- Active and low sedentary behaviour groups showed better results in all physical fitness tests than the others groups.

Article 3

- Participants in the active and low sedentary group consumed greater amounts of water and others beverages such as juice, milk, coffee, sport drink and alcohol drinks than the other groups.

- Physical fitness did not show significant differences between levels and fluid intake, except for distilled drinks.
- Participants met the German, Austrian and Swiss nutrition society's recommendations, being the mean of total liquid intake 1751 mL/day.

Article 4

- A significant relationship has been observed between micronutrients intake and physical fitness. However, supplementation should be considered for vitamins and minerals at risks.
- A significant inadequate intake below requirements for vitamin D, vitamin E and potassium has been displayed in both sexes, independently of their physical fitness level; although better micronutrient intake was observed in the high physical fitness group.

Article 5

- Sex has been identified as an important factor in the relationship between oxidative stress parameters with physical fitness.
- Greater malondialdehyde activity was observed in the high physical fitness level in females, while higher antioxidant enzyme activity measured by superoxide dismutase was observed in the high physical fitness level in males.
- Others lifestyles such as physical activity and sedentary behaviour micronutrient intake, age and body mass index showed association with oxidative stress parameters in both sexes.

Article 6

- High percentage of participants showed values out of reference range for vitamin D (68%), total cholesterol (59%), glucose (38%), uric acid (32%) low density lipoprotein cholesterol (28%) and total homocysteine (24%).
- Participants in the high physical fitness group presented better blood marker profile than in the low physical fitness group, concretely for total homocysteine, creatinine and lipid markers. Unexpectedly, participants in the low physical fitness group displayed less values out of the reference range for vitamin B₁₂ and triglycerides.

Article 7

- The physical fitness index and blood index have shown to be independent between them and both health markers should be included in a holistic health approach. Thus, we created a combined and novel classification according to physical fitness and blood markers called Total Index.
- Education was the principal predictor variable in the model, followed by physical activity and sedentary behaviour groups, waist to height ratio and age. Additionally, several associations were found between this classification and socioeconomic status, abdominal adiposity, anxiety and pain.

6.2 Practical Conclusions

- New reference ranges might be developed considering physical fitness level, specifically in older adult populations.
- To add vitamin blood markers at risk into the biochemical routine.
- Supplementation should be prescribed in this kind of population. Additionally, hydration recommendations should contemplate physical activity and fitness level.
- Nutritional and training programs should be developed jointly for older populations.
- In order to add life to years, to reduce health-care costs, to prevent and reduce diseases and to improve quality of life in older adults, the evaluation of physical fitness and activity, time spent sitting, nutrient and hydration intake and status, health and aging biomarkers, oxidative stress parameters and social environment may be mandatory in our health care system.

6.3 Future Research Lines

- A case-control complete profile study (biochemical, vitamins, oxidative stress parameters, physical fitness, nutrition, body composition) of extreme active and fit people.
- Further studies are required in females according to their physical activity level and sedentary behaviour and its relationship with physical fitness.
- To obtain reliable hydration markers in older adults and to investigate the association with physical fitness.

- To examine the role of physical activity and sedentary behaviour with oxidative stress parameters.
- To get deeper into the association between nutrient intake and blood markers.
- To elucidate and provide different measurements of ongoing optimal health and reliable predictors of biological aging.
- To replicate the project in a representative sample of Spanish older participants and follow them longitudinally.

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APPENDIX

APPENDIX 1

PROTOCOLO GENERAL

1.- Objetivos del estudio

Estudiar si existe una asociación entre la actividad física y los siguientes parámetros en personas mayores:

- (1) Estado de condición física y salud (cuestionario y pruebas físicas),
- (2) Hábitos alimentarios (cuestionario),
- (3) Estado de hidratación (cuestionario y biomarcadores),
- (4) Estado de vitamina B₁₂ (biomarcadores),
- (6) Densidad ósea, solo en Madrid,
- (7) Eje tiroideo (biomarcadores),
- (8) Oligoelementos y metales pesados (uñas),
- (9) Estrés oxidativo (biomarcadores).

2.- Población a estudio**2.1.- Definición y clasificación de los sujetos**

Los sujetos serán voluntarios (sin azar), mayores de 55 años y se dividirán en 3 grupos en función de la actividad física/deportiva realizada a lo largo de los últimos años. La mitad de los sujetos serán reclutados por la Universidad Politécnica de Madrid (UPM) y la otra mitad por la Universidad de las Islas Baleares (UIB). Se intentará obtener un número parecido de hombres y de mujeres.

Grupo N = No activos. Son sujetos que han realizado ≤ 2 horas/semana y 1 día por semana, de actividad física en los últimos 5 años ($n = 200$). Serán reclutados en clubs sociales y municipales.

Grupo A = Activos. Son sujetos que han realizado ≥ 4 horas/semana de actividad física y esto repartido en un mínimo de 3 días por semana, en los últimos 5 años ($n = 100$). Serán reclutados en federaciones de deporte y clubes de deporte: tenis, golf, atletismo, natación, esquí.

Grupo I = Intermedios. Son sujetos que no se puedan catalogar ni en el grupo N ni en el grupo A ($n = 100$). Serán reclutados en clubs sociales y municipales.

2.2.- Criterios de inclusión y exclusión**Inclusión:**

Se han incluido en el estudio todos aquellos hombres y mujeres mayores de 55 años, sin enfermedad cardiovascular documentada y que presenten además:

- Diabetes Mellitus tipo 2 o que reúnan tres o más de los siguientes factores:
- Tabaquismo (Fumadores de más de 1 cigarrillo al día).
- Hipertensión arterial: sujetos con presiones arteriales superiores o iguales a 140/90 mm Hg sin tratamiento o aquellos que sigan tratamiento hipotensor independientemente de sus cifras tensionales.
- Hipercolesterolemia: sujetos con cifras de LDL-colesterol > 160 mg/dl sin tratamiento o aquellos que sigan un tratamiento hipolipemiante independientemente de sus cifras de LDL-colesterol.
- Cifras de HDL-colesterol < 40 mg/dl, con o sin tratamiento hipolipemiante*.
- Sobrepeso u Obesidad (Índice de Masa Corporal > 25 kg/m²).

- Historia familiar de cardiopatía isquémica precoz (familiares de primer orden varones < 55 años o mujeres < 65 años).

* *En caso de HDL-colesterol > 60 mg/dl, se restará uno al número total de factores de riesgo vascular.*

Exclusión:

No deberán presentar las siguientes condiciones:

- Sujetos menores de 55 años.
- Sujetos institucionalizados.
- Enfermedad grave que limite su participación en las pruebas de condición física.
- Enfermedad mental que no permita al sujeto contestar a los cuestionarios del estudio.
- Pacientes que hayan recibido fármacos en fase de investigación durante el último año.

3.- Pruebas de condición física

Se utilizará el protocolo establecido por Rikli y Jones, adaptado por el Dr. Agustín Meléndez. Se seleccionan las siguientes 9 pruebas a realizar:

- (1) Test de equilibrio estático (mantenerse en el sitio sobre un pie).
- (2) Test de fuerza de agarre con dinamómetro (esta prueba es adicional al protocolo de Rikli y Jones): Para este test de fuerza de manos se utiliza un dinamómetro digital Takei TKK 5401. Se realizan dos intentos por cada mano, con el participante en posición de pie, y los brazos bajados y pegados al cuerpo. A continuación se realizan los mismos intentos pero esta vez el participante se encuentra sentado en una silla.
- (3) Test de fuerza de las extremidades inferiores (levantarse y sentarse en una silla).
- (4) Test de fuerza para las extremidades superiores (flexiones de brazos).
- (5) Test de flexibilidad de las extremidades inferiores (sentados y tocarse la punta del pie).
- (6) Test de flexibilidad de las extremidades superiores (rascarse la espalda).
- (7) Test de agilidad (test de levantarse, caminar 2"45 m y volver a sentarse).
- (8) Test de velocidad de la marcha (caminar deprisa 30 metros).
- (9) Test de resistencia aeróbica (caminar durante 6 minutos).

Los resultados se anotan en la hoja de recogida de datos de las pruebas de condición física.

4.- Medidas antropométricas

El resultado de las medidas se ha de anotar en la misma hoja de recogida de datos de las pruebas de condición física. Estas medidas se han de realizar antes del resto de la batería de pruebas físicas del protocolo de Rikli y Jones. Medidas a realizar:

- Talla.
- Perímetro de cintura y cadera.
- Tensión arterial.
- Composición corporal (Tanita BC-418).

5.- Análisis de muestras de sangre a realizar

En el laboratorio de la UPM se realizarán el hemograma y la bioquímica de rutina de los sujetos de Madrid, y también las pruebas de biomarcadores de vitamina B₁₂ y de hidratación de los sujetos tanto de la UPM como de la UIB. La UIB realizará las pruebas de bioquímica de rutina y hemograma de sus propios sujetos.

La preparación de las muestras para la determinación de parámetros de estrés oxidativo se realiza en el laboratorio de la UPM (para sujetos de Madrid). Sin embargo, el análisis propiamente dicho se realiza en la UIB, a quién hay que remitir el preparado de las muestras preparadas en la UPM.

La determinación de los elementos traza y metales pesados será realizado por la Universidad de Delft (Holanda).

Parámetros bioquímicos a determinar:

Hemograma	Bioquímica rutina	Biomarcadores B ₁₂ y otros	Biomarcadores hidratación	Estrés oxidativo
Hb,Hto ,RBC,RETIC ,LYM,BASO ,NEU, MONO,EOS, PLT,NRBC, IRF	Na, K, Cl ,GOT, GPT,GGT,TG, LDL, LDL, VLDL,Albúmin a ,Proteínas T, Colesterol, Ácido úrico, Hierro, ferritina	Cobalamina, Folato sérico y RBC, Homocisteína, TSH, T4L, vitamina D	Osmolalidad suero, Creatinina, Cistatina C, Copeptina (CT Pro AVP), Aldosterona	Nitrotirosina, Índice de carbonilos,malonil dialdehído,glutation peroxidasa,glutation reductasa, C catalasa,mieloperoxidasa, xantina oxidasa, tioredoxina reductasa, selenoproteína P, metolotionina, SOD (Mn, Cu/Zn/EC), isoformas, ascorbato, tocoferoles, glutation oxidado/reducido, nitrito y nitrato

6.- Preparación de muestras para evaluación de estrés oxidativo

Las soluciones obtenidas al final se almacenarán a -80°C en la UPM hasta su envío al laboratorio de la UIB.

El Ficoll y el Tripure se compran directamente, mientras que el PBS y el RIPA pueden prepararse de la siguiente forma:

PBS (1L): 8g de NaCl

0.2 g KCl

0.923 g Na₂HPO₄

0.2 g KHPO₄

Ajustar pH a 7.4

RIPA (5X): Tris-HCl, pH 8, 1M : 12.5 mL

$$20\text{mL} \times \frac{1\text{L}}{1000\text{mL}} \times \frac{1\text{mol}}{1\text{L}} \times \frac{157,6\text{g}}{1\text{mol}} = 3,152\text{g}$$

2.2 g NaCl

2.5 g IGEPAL

1.25 g ácido deoxicólico (difícil de disolver)

2.5 mL SDS 10% (0.3 g en 3 mL) Enrasar a 50 mL

7.- Preparación de las muestras de uñas

La Universidad de Delft realizará la determinación de Zn, Se, Cr y Hg mediante la técnica de activación de neutrones. Una vez recogidas las muestras en la UPM, se mandarán a Delft. Se entregará a cada participante una bolsita de plástico para que guarde las uñas cortadas de todos los dedos de los pies. Se necesita un mínimo de 25 mg. El participante entregará la bolsita con la uñas cuando acuda al laboratorio de la UPM para realizar la extracción de sangre. Para las mujeres, pedirles que antes de que se corten las uñas eliminen el esmalte, si es que las tienen pintadas.

8.- Hábitos alimentarios, hidratación y práctica de actividades físicas

Para ello se utilizarán los cuestionarios de frecuencia de alimentos (PREDIMED), recordatorio de 24 horas (UIB), hidratación (UPM), actividad física (MINNESOTA) e información general y hábitos (EXERNET).

9.- Pruebas de densitometría ósea (DXA)

El día del examen el participante puede alimentarse normalmente. Sin embargo, no deberá ingerir suplementos con calcio durante al menos 24 horas antes del examen. Deberá utilizar ropa cómoda y suelta, evitando prendas que tengan cierres, cinturones o botones de metal. Se deben sacar los objetos tales como llaves o billeteras que pudieran encontrarse en el área a examinar. Se le puede solicitar que se quite toda o parte de su vestimenta y que utilice una bata durante el examen. También se le puede solicitar que se quite joyas, lentes y cualquier objeto de metal o vestimenta que pueda interferir con las imágenes de rayos X.

El participante tiene que informar al operador de la densitometría ósea si recientemente ha tenido un examen con bario o le han inyectado un medio de contraste para una tomografía axial computada (TAC) o una radioisotopía, ya que habría que esperar 15 días antes de hacer la prueba. Por último, las mujeres siempre deben informar al operador si existe la posibilidad de embarazo.

APPENDIX 2

“Determinantes de riesgo de primeros eventos cardiovasculares. Un estudio coordinado de casos y controles de la cohorte PREDIMED. Subproyecto 02. Antioxidantes y estrés oxidativo”. Número de proyecto: FIS PI11/01791.

Estimado amigo:

El proyecto en el cual le invitamos a participar es un estudio centrado en averiguar la influencia de varios factores sobre el equilibrio oxidativo y las posibles interrelaciones que puedan existir entre dichos factores en personas. Estos factores son la actividad física desarrollada en los últimos, el estado de vitamina B₁₂, los niveles de oligoelementos, los hábitos de alimentación e hidratación y el remodelado óseo.

¿Cuál es el interés de este estudio?

La vitamina B₁₂ es la vitamina más deficitaria en las personas mayores, y su deficiencia se relaciona con diversas molestias como dolor u hormigueo de piernas, pérdida de coordinación en manos y piernas, pérdida de memoria, un cierto tipo de anemia, entre otros. Si esta deficiencia no es detectada a tiempo, se produce un empeoramiento de los síntomas señalados anteriormente, dando lugar incluso a demencia senil.

Los oligoelementos juegan un papel fundamental en la salud y el bienestar del individuo. Participan en toda una serie de reacciones fundamentales en el cuerpo humano, como pueden ser la respiración o la expresión de genes. Para que los oligoelementos cumplan su papel han de estar en el cuerpo en concentraciones óptimas. Un déficit o un exceso de oligoelementos pueden alterar los procesos biológicos, por lo que es importante controlar sus niveles. Las uñas de los pies es el lugar del cuerpo donde más se acumulan y por tanto, son la mejor muestra donde analizar estos niveles. En nuestro estudio nos ocuparemos de tres oligoelementos: el cromo (Cr), el zinc (Zn) y el selenio (Se).

Está demostrado que seguir una dieta sana, tener siempre una hidratación conveniente y realizar una actividad física regular a lo largo del tiempo son fundamentales para la buena salud del organismo, reduciendo el riesgo de algún evento cardiovascular grave y de problemas de sobrepeso u obesidad.

Por último, el equilibrio oxidativo es el balance final entre moléculas oxidantes y moléculas antioxidantes, formadas a partir del oxígeno obtenido de la respiración. Es importante que este balance sea el correcto para reducir el riesgo de enfermedades, algunas de ellas graves, como las enfermedades cardiovasculares. Nuestra hipótesis es que los factores anteriormente citados tienen una estrecha relación con el equilibrio oxidativo.

¿Cómo se desarrolla el estudio?

Sus hábitos de alimentación e hidratación, su actividad física realizada en los últimos años y su estado general serán evaluados mediante cuestionarios. Posteriormente, tendrán que realizar una serie de pruebas físicas sencillas, se les tomará una muestra de sangre (40 ml) y se les realizará una densitometría ósea.

En principio el estudio se realizará en dos fases. El primer día les tomaremos la muestra de sangre y les haremos una parte de los cuestionarios. Este día les entregaremos una bolsita de plástico que se llevarán a casa y donde tendrán que depositar las uñas cortadas de los dedos de los pies. El segundo día (2-3 semanas después) se rellenarán el resto de cuestionarios y se realizará una batería de pruebas físicas muy sencillas y la composición corporal. También tendrán que traer la bolsita con las uñas de los pies.

Al final del estudio recibirán un informe completo con todos los resultados. Estamos a su entera disposición para aclarar cualquier duda que pueda surgir, incluso durante el transcurso de la investigación. Por ello, al final de esta carta les indicamos el teléfono de contacto y correo electrónico del Investigador Principal.

¿Por qué participar en este estudio?

Los avances científicos sólo son posibles gracias al entusiasmo y apoyo de muchas personas, sobre todo voluntarios que se prestan a participar en este tipo de estudios. El estudio que se plantea se va a realizar de este modo por primera vez en España. Por lo tanto, somos pioneros y necesitamos su apoyo para poder seguir adelante.

Este proyecto es muy importante para mejorar la salud de la población mayor. Una de las máximas que citamos con frecuencia en nuestro grupo de investigación es la de la Organización Mundial de Salud “no sólo añadir años a la vida sino vida a los años”. Para ello, es fundamental llegar a mayor con una salud lo mejor posible, en plena posesión de las capacidades físicas y mentales. Estudios como el nuestro pretenden profundizar en estos aspectos, con el fin de mejorar la vida “funcional” de las personas.

Este proyecto se lleva a cabo siguiendo escrupulosamente toda la legislación vigente en torno a la realización de estudios de investigación en humanos. Para poder participar en el estudio, es necesario que firme la hoja de consentimiento informado que le habremos entregado. Usted tiene el derecho de dejar de participar en el estudio en cualquier momento, sin alegar razones y sin que ello repercuta en ningún modo en ningún aspecto relacionado con su vida personal.

Reciba un cordial saludo,

Prof. Marcela González-Gross

Investigador Principal

Dpto. Salud y Rendimiento Humano, Facultad CC Actividad Física y Deporte, Universidad Politécnica de Madrid. Avda. Martín Fierro, 7; 28040 Madrid.

Teléfono de contacto: 913 364 039. Correo: marcela.gonzalez.gross@upm.es

APPENDIX 3

Consentimiento informado por escrito del voluntario

“Determinantes de riesgo de primeros eventos cardiovasculares. Un estudio coordinado de casos y controles de la cohorte PREDIMED. Subproyecto 02. Antioxidantes y estrés oxidativo”. Número de proyecto: FIS PI11/01791.

El Instituto de Salud Carlos III ha mostrado su interés en apoyarnos en la realización de un estudio científico de gran importancia para la salud de las personas mayores. Este estudio se va a llevar a cabo siguiendo escrupulosamente la legislación vigente y ha sido aprobado por el Comité de Ética de la Universidad Politécnica de Madrid.

Esta es la razón por la cual les vamos a realizar un análisis de sangre (40 ml) para determinar niveles de biomarcadores en sangre, marcadores genéticos relacionados con la condición física, el contenido de oligoelementos en las uñas de los pies y una densitometría ósea. Por último, le haremos también algunas preguntas sobre la asiduidad e intensidad de la actividad física que realice y los hábitos de alimentación y consumo de bebidas que sigue.

En el supuesto que algún resultado de las pruebas realizadas fuese patológico, el Dr. Javier Rojo González, médico del INEF asignado a este estudio (telf. contacto 91 336 40 26), se pondría en contacto con Ud. y posteriormente le remitiría a su médico de cabecera.

Por tanto, le rogamos, una vez leída la carta adjunta, que firme el siguiente consentimiento informado. Este consentimiento se expide por duplicado, con el fin de que Ud. guarde una copia y el equipo investigador otra, en un sobre cerrado.

Yo, (nombre y apellidos del voluntario)

He leído la hoja de información que se me ha entregado.

He podido hacer preguntas sobre el estudio.

He recibido suficiente información sobre el estudio.

He hablado con: (nombre del investigador)

Comprendo que mi participación es voluntaria.

Comprendo que para el estudio he de donar una muestra de sangre.

Comprendo que puedo abandonar el estudio en cualquier momento por decisión propia.

Presto libremente mi conformidad para participar en el estudio.

Firma del voluntario

Firma del investigador

Madrid, a de de 201

APPENDIX 4

HOJA DE REGISTRO BATERIA DE PRUEBAS FISICAS

Nombre y apellidos: _____

Fecha de la encuesta: _____

Localidad: _____

Código: _____

Investigador responsable: _____

	SI	NO
Firma consentimiento informado		
Rellena cuestionario		

Tensión arterial	Toma 1	Toma 2	Toma 3	Valor final

Sangre recogida	SI	NO

Perímetros (cm)	Toma 1	Toma 2	Toma 3
Cintura			
Cadera			

Talla (cm)	Toma 1	Toma 2

Tanita realizada	SI	NO

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BATERÍA DE TEST DE CONDICIÓN FÍSICA

Equilibrio	1ª Evaluación		2ª Evaluación	
	Derecha	Izquierda	Derecha	Izquierda
Equilibrio sobre una pierna				

Fuerza y Presión manual de pie	1ª Evaluación		2ª Evaluación	
	Derecha	Izquierda	Derecha	Izquierda
Dinamometría manual (kg)				

Fuerza y Presión manual sentado	1ª Evaluación		2ª Evaluación	
	Derecha	Izquierda	Derecha	Izquierda
Dinamometría manual (kg)				

Fuerza miembro inferior	Evaluación
Sentarse y levantarse (30 seg)	

Fuerza miembro superior	Evaluación
Flexiones de codo con mancuernas (30 seg)	

Tests de Flexibilidad de piernas	1ª Evaluación	2ª Evaluación
Inclinación sobre la pierna (cm)		

Tests de Flexibilidad de brazos	1ª Evaluación	2ª Evaluación
Rascarse la espalda (cm)		

Tests de agilidad	1ª Evaluación	2ª Evaluación
Rodear un cono (seg)		

Velocidad	1ª Evaluación	2ª Evaluación
Test velocidad 30m		

Resistencia	Evaluación
Test de los 6 min	

Firma del encuestador:

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APPENDIX 5

Determinantes de riesgo de primeros eventos cardiovasculares. Un estudio coordinado de casos y controles de la cohorte PREDIMED, Subproyecto 02. Antioxidantes y estrés oxidativo.

Nombre: _____ Lugar de Madrid
evaluación: _____
Edad: 77 años Fecha de 31/01/2014
evaluación: _____

EVALUACIÓN DE LA CONDICIÓN FÍSICA

La condición física se define como la habilidad que tiene una persona para realizar actividades de la vida diaria con vigor. Un buen nivel de condición física se asocia con un menor riesgo de enfermedades crónicas y muerte prematura. Sus principales componentes son: capacidad cardiorrespiratoria, capacidad músculo-esquelética y capacidad motora. En personas mayores es importante mantener un nivel adecuado de condición física para asegurarse la independencia al realizar las actividades de la vida cotidiana.

Los valores que se obtuvieron tras su evaluación fueron los siguientes:

		Muy bajo	Bajo	Medio	Alto	Muy alto
Equilibrio	115 s					X
Fuerza piernas	12 rep		X			
Fuerza brazos	Der: 16 rep			X		
Flexibilidad piernas	Der: 6 cm Izq: 1 cm				X	
Flexibilidad brazos	Der: -3 cm Izq: -17 cm			X		
Agilidad	5 s					X
Velocidad	16 s					X
Resistencia	572 m				X	

Hay algunos componentes de su condición física que se podrían mejorar. A continuación exponemos unas pautas que podrían ayudarle a mejorar su condición física. Para mejorar la fuerza, se recomiendan ejercicios de fortalecimiento muscular como pueden ser subir escaleras o levantar peso con los brazos. Para mejorar su flexibilidad, conviene realizar estiramientos musculares con regularidad.

ACKNOWLEDGMENTS/ AGRADECIMIENTOS

Dicen que si crees, creas...4 años de una etapa maravillosa en la que no he dejado de aprender y crecer, un camino largo y duro sabiendo que detrás de cada logro, hay otro desafío. A todas las personas que han estado a mi lado este tiempo, gracias.

A mis padres, Alfonso y Julia, por su amor infinito y por su constante e incansable ayuda. Por enseñarme que con esfuerzo, humildad, trabajo, responsabilidad y constancia todo se consigue. Por ser claro ejemplo de todos estos valores que han hecho que sea su fiel reflejo, os debo todo. ¡Sois mi vida!.

A mi “xaxe” Pablo, por ser el mejor regalo que la vida me ha dado. Eres parte fundamental de mí y siento un tremendo orgullo de ti. Gracias por todo lo que me has enseñado y guiado, por nuestros grandes momentos juntos. Ayer, hoy, mañana y siempre, te quiero hermano. A Cinta, tu compañera de viaje, gracias por estar siempre ahí.

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A mi tía Rosali, por ser mi “mami madrileña” porque sin ti no hubiese sido posible llegar hasta aquí. A mi tía Mari, por mantenerme bien “nutrida”. A mi prima Nere y mis mellis (Ángel y Martina); ahora no os dais cuenta pero vosotros me habéis regalado tardes de infinita felicidad. Hicisteis posible que sonriese en tiempos difíciles.

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A toda mi maravillosa familia Ugarriza, no sé qué sería de mí sin vosotros. Gracias por confiar ciegamente en mí y darme todo lo que en vuestras manos ha estado. Os quiero.

Gracias a toda mi gran familia Aparicio, especialmente a mi tío Javi; tu ejemplo nos hace que nunca caigamos. Os quiero.

A mi querida familia política, Pascual, Angelita, Natalia y Ote. Especialmente a Angelita, por ser ejemplo absoluto de superación, nunca nos dejaste caer y nunca te faltó una sonrisa. Aprendo tanto a vuestro lado, gracias por todo...Y a Macu y familia, por ser tan estupendos.

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A mis amigos “madrileños”, alegrasteis y amenizasteis mis días, muchas gracias.

A mi directora de tesis, Dra. Marcela González-Gross, “madre de tesis”, por creer en mí y brindarme la oportunidad de poder trabajar a tu lado. Por enseñarme continuamente, por permitirme crecer, profesional y personalmente. Se necesitan personas que crean en los jóvenes, capaces de dedicarles tiempo y guiarles en su camino. Por todo ello, te estoy muy agradecida, ¡muchísimas gracias por todo!.

A mi “padre de tesis”, Dr. Gonzalo Palacio Le Blé, gracias por la confianza que depositaste en mi desde el inicio. Nunca te faltó una palabra de aliento y con tus consejos y sabías palabras me diste la paz que se necesita en este camino. Gracias por hacerme crecer, a tu lado he madurado profesionalmente y todavía más importante, personalmente. Esta tesis doctoral es fruto de tu constante ayuda; nuevamente gracias.

Al grupo ImFINE, por ser parte imprescindible de este proceso. A Agustín Meléndez, gracias por ser un libro abierto, he aprendido mucho contigo. A Raquel Pedrero, por dejarme aprender de ti, eres una gran profesional y mejor persona, muchas gracias. A Raquel Luzardo, por ser parte fundamental de este proceso y por dejar una huella en mí, sigue luchando por tus sueños. A Sergio Calonge, porque hemos compartido muchas muchas horas juntos, momentos felices y tiempos difíciles, pero siempre hemos conseguido salir. Por saber escuchar y ayudar, por tu entera disposición y tu buen humor, por tu generosidad, gracias. Ánimo, todo llega y todo pasa, para lo que necesites ahí estaré. A David Cañada, gracias por haberme permitido trabajar a tu lado. A mis amigos con lo que inicié esta etapa y que he tenido la gran suerte de poder seguir

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Al grupo NUCOX, de la Universidad de las Islas Baleares y liderado por Josep A. Tur, gracias por dejarme forma parte de este proyecto.

A Inge Huybrechts, mi supervisora durante la estancia en Lyon. Gracias por dejarme aprender de ti, por brindarme la oportunidad de trabajar a tu lado, admiro tu forma de ser y trabajar. Solo puedo estar agradecida. To Inge Huybrechts, my supervisor during

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No puedo terminar los agradecimientos sin dedicar unas palabras a quienes hicieron posible el proyecto, a todos los participantes, muchísimas gracias por vuestra colaboración voluntaria, sin vosotros no hubiera sido posible.

A todos los profesores que marcaron mi camino y de los que me siento orgullosa: Fito, Raúl, Enrique,... ¡¡gracias!!.

MUCHAS GRACIAS A TODOS

“Voici mon secret. Il est très simple: on ne voit bien qu'avec le cœur.
L'essentiel est invisible pour les yeux”.
#LePetitPrince #AntoinedeSaint-Exupéry

SUMMARIZED CV/CURRÍCULUM VITAE ABREVIADO

Educational background

- M.Sc. in Physical Activity and Sport Sciences. University of Miguel Hernandez of Elche. 2007-2012.
- Master Degree in Sports Performance and Health. University of Miguel Hernandez of Elche. 2012-2013.
- Master Degree in Secondary Education Teaching, Vocational Training and Language Teaching. University of Miguel Hernandez of Elche. 2012-2013.
- Specific Master Degree in Nutrition and Food: Principles and Trends. University of Barcelona. 2016-2017.
- Massage applied to Physical Activity and Sport Title. Universidad Politécnica de Madrid. 2016.
- Instructor of summer school. University of Miguel Hernandez of Elche. 2010.
- Instructor of swimming. University of Miguel Hernandez of Elche. 2009.

Grants and awards received

- Competitive grant: Meetings Excellence in Public Health Research. CIBER of Epidemiology and Public Health, CIBERESP. Instituto de Salud Carlos III, Spain. Spanish Ministry of Economy, Industry and Competitiveness. 17/07/2017.
- 2nd National Sports Medicine Award: "Effect on the physical condition of an interdialysis exercise program in patients with chronic kidney disease". University of Oviedo. Oviedo. 12/2016.
- Competitive grant: "English Language Immersion Course". Menéndez Pelayo International University. Education, Culture and Sport Spanish Ministry. 12/2016.
- Best oral communication: "Fitness cardiovascular, body composition and adiposity in Spanish elderly". XI Spanish Society of Community Nutrition. Zaragoza. 09/2016.
- Competitive grant: XIV Internationalization Grant for PhD Student from Social Council of the Universidad Politécnica de Madrid. Stay at International Agency for Research on Cancer (IARC). World Health Organization (WHO). Lyon (France). 05/2016.

- Best oral communication: “Impact of physical activity and sedentarism on hydration status and liquid intake in Spanish older adults”. II International Congress and IV National Hydration. Toledo. 12/2015.
- Competitive grant: “English Language Immersion Course”. Menéndez Pelayo International University. Education, Culture and Sport Spanish Ministry. 08/2015.
- “Promising Best Young Researcher Grant”: Asociación entre homocisteína, folato y vitamina B₁₂ con la condición física en personas mayores de 55 años. Spanish Society of Nutrition (SEÑ). Sevilla. 03/2015.
- Fund as fellowship from the Fundación Renal Iñigo Álvarez de Toledo. 10/2014 (9 months).
- Best academic performance: “5 stars student to the comprehensive university training”. University Miguel Hernandez of Elche. Elche. 04/2013.

Stays abroad

- International Agency for Research on Cancer (IARC). World Health Organization (WHO). Lyon, Francia. 9 May-19 August 2016 (3 months and 10 days).
- Northern Ireland Centre for Food & Health (NICHE), School of Biomedical Sciences, University of Ulster. United Kingdom. June-July, 2015 (3 weeks).

Teaching and invited lectures

- **Official teaching: Actividades Acuáticas para la Salud y, Nutrición, Deporte y Valoración de la Condición Física** (120 h). Faculty of Physical Activity and Sport-INEF, Universidad Politécnica de Madrid. 2015-2017.
- **Invited lectures: “Validación de cuestionarios”** (3 h). Faculty of Physical Activity and Sport-INEF, Universidad Politécnica de Madrid. April, 21th 2017.
- **Invited lectures: “Beneficios y riesgos del ejercicio en el medio acuático”, Actividades Acuáticas para la Salud** (2 h). Faculty of Physical Activity and Sport-INEF, Universidad Politécnica de Madrid. September, 16th 2014.
- **Invited lectures: “Aquagym. Entrenamiento cardiovascular. Ejercicios básicos”, Actividades Acuáticas para la Salud** (2 h). Faculty of Physical

Activity and Sport-INEF, Universidad Politécnica de Madrid. September, 30th 2014.

- **Invited lectures: “Formas de desarrollo, mejora y mantenimiento de la condición física del individuo mediante el Fitness Acuático”, Actividades Acuáticas para la Salud (2 h).** Faculty of Physical Activity and Sport-INEF, Universidad Politécnica de Madrid. October, 14th 2014.
- **Invited lectures: “Formas de desarrollo, mejora y mantenimiento de la fuerza muscular del individuo mediante el Fitness Acuático”, Actividades Acuáticas para la Salud (2 h).** Faculty of Physical Activity and Sport-INEF, Universidad Politécnica de Madrid. October, 23th 2014.
- **Invited lectures: “Programas especiales. Consideraciones básicas para el trabajo en el agua con mayores”, Actividades Acuáticas para la Salud (2 h).** Faculty of Physical Activity and Sport-INEF, Universidad Politécnica de Madrid. October, 28th 2014.
- **Invited lectures: “Recursos y procedimientos metodológicos para el desarrollo de la resistencia mediante el Fitness Acuático”, Actividades Acuáticas para la Salud (2 h).** Faculty of Physical Activity and Sport-INEF, Universidad Politécnica de Madrid. November, 18th 2014.

Research projects

- **Title: “Evolución de la condición física, composición corporal y fragilidad en personas mayores de 65 años. Mediación de la vitamina D y efectos de un programa de ejercicio: Estudio longitudinal EXERNET”.** **Project number:** DEP2016-78309-R. **Funding:** Ministerio de Economía y Competitividad y por la Comisión Europea. **Duration:** 2017-2019. **Principal researcher:** Germán Vicente Rodrigo (Universidad de Zaragoza).
- **Title: Efecto del ejercicio físico controlado en el paciente en hemodiálisis. Estudio de marcadores emergentes sobre el pronóstico de la enfermedad renal crónica.** **Project number:** P2016/RM10. **Funding:** Cátedra Real Madrid-Universidad Europea de Madrid. **Duration:** 2016-2017. **Principal researcher:** Catalina Santiago Dorrego (Universidad Europea de Madrid).
- **Title: PREDIMED+DM. Effect of a hypocaloric Mediterranean diet and physical activity promotion on the prevention of type 2 diabetes mellitus in participants with the Metabolic Syndrome.** **Project number:** PI14/00636.

Funding: Instituto de Salud Carlos III. Ministerio de Sanidad, Servicios Sociales e Igualdad. Duration: 2015-2017. Principal researcher: Josep Antoni Tur Mari (Universidad de las Islas Baleares).

- Title: **Proyecto piloto de implantación de ejercicio físico durante la hemodiálisis**. Project number: P141115303. Funding: Fundación Renal Iñigo Álvarez de Toledo. Duration: 2014-2016. Principal researcher: Marcela González-Gross (Universidad Politécnica de Madrid).
- Title: **Determinantes de riesgo de primeros eventos cardiovasculares. Un estudio coordinado de Casos y Controles anidado de la cohorte PREDIMED. Subproyecto 02. Antioxidantes y estrés oxidativo**. Project number: PI11/01791. Funding: Instituto de Salud Carlos III. Ministerio de Sanidad, Servicios Sociales e Igualdad. Duration: 2013-2016. Principal researcher: Josep Antoni Tur Mari (Universidad de las Islas Baleares).

Scientific publications apart from thesis publications

- **Aparicio-Ugarriza R**, Cuenca-García M, Gonzalez-Gross M, Julián C, Moreno LA, Breidenassel C, Kersting M, Arouca A, Michels N, Mouratidou T, Manios Y, Dallongeville J, Gottrand F, Widhalm K, Kafatos A, Molnar D, De Henauw S, Gunter M, Huybrechts I, on behalf of the HELENA consortium. Relative validation of adapted Mediterranean Diet Scores for Adolescents by comparison with nutritional biomarkers and nutrient and food intakes: the HELENA study. Br J Nutr (submitted). (JCR: 3.311).
- Moradell A, Santaliestra-Pasías AM, **Aparicio-Ugarriza R**, Bertalané Szommer A, Forsner M, González-Gross M, Gutierrez A, Kafatos A, Manios Y, Michels N, Sjöström M, Vanhelst J, Widhalm K, Huybrechts I, and Moreno LA, on behalf of the HELENA study group. Physical activity and sedentary behaviour levels were related with food consumption in European adolescents? The HELENA study. Br J Nutr (submitted). (JCR: 3.311).
- Barker AR, Gracia-Marco L, Ruiz J, Castillo MJ, **Aparicio-Ugarriza R**, González-Gross M, Kafatos A, ndroutsos O, Polito A, Molnar D, Widhalm K and Moreno LA. Physical activity, sedentary time, TV viewing, physical fitness and cardiovascular disease risk in adolescents: The HELENA study. Int J Cardiol (first reviewed). (JCR: 4.638).

- **Aparicio-Ugarriza R**, Cañada D, Ruiz E, Ávila JM, Aranceta-Bartrina J, Gil A, Ortega RM, Serra-Majem L, Varela-Moreiras G, González-Gross M. Active commuting, physical activity, and sedentary behaviors in children and adolescents from Spain: findings from the ANIBES study. BMC Public Health (first reviewed). (JCR: 2.209).
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- Sureda A, Bibiloni MM, Julibert A, **Aparicio-Ugarriza R**, Palacios G, Pons A, González-Gross M, Tur JA. Trace element contents in toenails are related to regular physical activity in old adults. PLoS One. 2017 Oct 6;12(10):e0185318. (JCR: 3.057).
- **Aparicio-Ugarriza R**, Rumi C, Luzardo-Socorro R, Mielgo-Ayuso J, Palacios G, Bibiloni MM, Julibert A, Argelich E, Tur JA, González-Gross M. Seasonal variation and diet quality among Spanish people aged >55 years. J Physiol Biochem (accepted). (JCR: 2.054).
- Bibiloni MM, Karam J, Emma Argelich, **Aparicio-Ugarriza R**, Pedrero-Chamizo R, Sureda A, González-Gross M, Tur JA. Sociodemographic, health and lifestyle determinants of physical condition in Spanish older adults. BMC Geriatrics (submitted). (JCR: 2.371).
- Bibiloni MM, Julibert A, Argelich E, **Aparicio-Ugarriza R**, Palacios G, Pons A, González-Gross M, Tur JA. Western and Mediterranean dietary patterns and physical activity and fitness among Spanish old adults. Nutrients 2017 Jul 6;9(7). pii: E704. doi: 10.3390/nu9070704. (JCR: 3.375).
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- Mielgo-Ayuso J, **Aparicio-Ugarriza R**, Castillo A, Ruiz E, Ávila JM, Aranceta-Bartrina J, Gil A, Ortega RM, Serra-Majem L, Varela-Moreiras G, González-

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 - **Aparicio-Ugarriza R**, Mielgo-Ayuso J, Benito PJ, Pedrero-Chamizo R, Ara I, Gonzalez-Gross M, EXERNET Study Group. Physical activity assessment in the general population; instrumental methods and new technologies. *Nutr Hosp*. 2015;31(s03):219-26. (JCR: 1.497).
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- Carretero-Krug A, Úbeda N, Montero A, Medina-Font J, Escobar-Toledo D, Arche-Navarro AV, **Aparicio-Ugarriza R**, Córdoba-Gómez A, González-Gross M, Varela G. Evaluación del estado nutricional en personal militar de vuelo del

Centro de Instrucción de Medicina Aeroespacial (CIMA). XVIII Symposium Nacional de Medicina Aeroespacial. Granada (Spain). November, 2017.

- **Aparicio-Ugarriza R**, López-Torres O, Díaz AE, Bibiloni MM, Julibert A, Palacios G, Tur JA, González-Gross M. A novel approach to analyse the relationship between physical fitness and biomarkers in older adults. IUNS 21st International Congress of Nutrition. Argentina (Buenos Aires). Ann Nutr Metab 2017; (suppl 2):1-1433.
- **Aparicio-Ugarriza R**, López-Torres O, Maroto-Sánchez B, Bibiloni MM, Julibert A, Palacios G, Tur JA, González-Gross M. Better micronutrient intake could be related to physical fitness in older adults? IUNS 21st International Congress of Nutrition. Argentina (Buenos Aires). Ann Nutr Metab 2017; (suppl 2):1-1433.
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- **Aparicio-Ugarriza R**, Luzardo-Socorro R, Palacios G, Bibiloni MM, Pons A, Tur JA, González-Gross M. Combined physical activity and sedentarism levels are related with different blood lipid profiles. 22nd annual Congress of the European College of Sport Science. ISSN 978-3-9818414-0-4. Essen (Germany). July, 2017.
- Vicente-Arche FC, Pedrero-Chamizo R, Vázquez-Rigueira P, Peñaranda A, **Aparicio-Ugarriza R**, Santiago C, Calonge S, Gómez-Gallego F, Palacios G, Diaz-Manjón R, Meléndez A, Barrios L, Botella A, González-Gross M, Pérez-Ruiz M. Effect of intradialysis exercise program on physical fitness in chronic kidney disease. 22nd annual Congress of the European College of Sport Science. ISSN 978-3-9818414-0-4. Essen (Germany). July, 2017.

- **Aparicio-Ugarriza R**, Luzardo-Socorro R, Palacios G, Bibiloni MM, Julibert A, Tur JA, González-Gross M. ¿Está la ingesta de macro y micronutrientes relacionada con la condición física de los españoles adultos? XVII Reunión de la Sociedad Española de Nutrición. IV Jornadas de Jóvenes Investigadores. Nutr Hosp 2016; 33(Supl.7):3-63. ISSN 0212-1611. Santiago de Compostela (Spain). November, 2016.
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- **Aparicio-Ugarriza R**, Luzardo-Socorro R, Palacios G, Bibiloni MM, Tur JA, González-Gross M. Combined physical activity and sedentarism levels are related with different blood lipid profile. Simposio EXERNET. Investigación en Ejercicio, Salud y Bienestar: “Exercise is Medicine”. Revista Andaluza de Medicina del Deporte. Cádiz (Spain). October, 2016.
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- **Aparicio-Ugarriza R**, Palacios G, Bibiloni MM, Julibert A, Tur JA, González-Gross M. Impact of physical activity and sedentarism on hydration status and liquid intake in Spanish older adults. II International and IV National Spanish HYDRATION Congress. Nutr Hosp. 2015;32(s02):32:21-22. (JCR: 1.497).
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- intake? II International and IV National Spanish HYDRATION Congress. *Nutr Hosp.* 2015; 32(s02):32:37. (JCR: 1.497).
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 - Luzardo-Socorro R, **Aparicio-Ugarriza R**, Mielgo-Ayuso J, Bibiloni MM, Sureda A, Julibert A, Tur JA, González-Gross M. Influencia de la estacionalidad en la ingesta de bebidas en mayores españoles. Proyecto FIT-MED. XVII Congreso Latinoamericano de nutrición (SLAN). Nutrición para el desarrollo sostenible. Libro de comunicaciones. Nutrición para el desarrollo sostenible (pág.154). Punta Cana (Santo Domingo). November, 2015.
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- **Aparicio-Ugarriza R**, Mielgo-Ayuso J, Luzardo-Socorro R, Maroto-Sánchez B, Seco R, Argelich E, Biblioni MM, Palacios G, Tur JA, González-Gross M. Impact of former performed physical activity on body composition and physical fitness in postmenopausal Spanish women. European Congress on Menopause and Andropause. Abstracts / Maturitas 81 (2015) 191–236. Madrid (Spain). June, 2015.
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- **Aparicio-Ugarriza R**. Sedentarism, nutritional status and exercise in adults over 55. A comparison between Madrid and Palma de Mallorca. III Jornadas de Jóvenes investigadores en nutrición. Sevilla (Spain). March, 2015.
- Luzardo-Socorro R, **Aparicio-Ugarriza R**, Maroto-Sánchez B, Marín-Puyalto J, Palacios G, González-Gross M. Valoración de la fuerza muscular en relación al consumo de bebidas en personas mayores españoles. VII International Symposium in Strength Training. Libro abstract VII Simposio Internacional de

- actualizaciones en entrenamiento de la fuerza, 2014: 82. ISSN 978-84-697-1880-3. Universidad Politécnica de Madrid. Madrid (Spain). December, 2014.
- **Aparicio-Ugarriza R**, Marín-Puyalto J, Luzardo-Socorro R, Maroto-Sánchez B, Tur JA, Palacios G. Adequacy of muscular mass estimations provided by bioimpedance analysis for the assessment of body composition in participants over 55. III World Congress of Public Health Nutrition. International Journal of Community Nutrition 2014, 0 (suppl); 2014; 122. ISSN 2386-673X. Las Palmas de Gran Canaria (Spain). November, 2014.
 - Maroto-Sánchez B, Luzardo-Socorro R, **Aparicio-Ugarriza R**, Palacios G, Diaz AE, González-Gross M. Fluid intake, biomarkers and body composition differences between physically active and non-active elderly people. III World Congress of Public Health Nutrition. International Journal of Community Nutrition 2014, 0 (suppl); 2014; 123. ISSN 2386-673X. Las Palmas de Gran Canaria (Spain). November, 2014.
 - **Aparicio-Ugarriza R**, Marín-Puyalto J, Luzardo-Socorro R, Butragueño J, Palacios G, Tur JA, González-Gross M. Causes of deviations on bioelectric impedance assessment versus dual X-ray absorptiometry in women over 55. World Conference on Kinanthropometry. Arch Med Deporte 2014; 31: 194. ISSN: 0212-8799. Murcia (Spain). July, 2014.
 - Marín-Puyalto J, **Aparicio-Ugarriza R**, Luzardo-Socorro R, Maroto-Sánchez B, Palacios G, Tur JA, González-Gross M. Comparison between 2-compartment and 3-compartment bioimpedance analysis estimations of body composition in a population over 55. World Conference on Kinanthropometry. Arch Med Deporte 2014; 31: 194. ISSN: 0212-8799170-199. Murcia (Spain). July, 2014.
 - **Aparicio-Ugarriza R**, Marín-Puyalto J, Luzardo-Socorro R, Mielgo-Ayuso J, Butragueño J, Palacios G, Tur JA, González-Gross M. Validez de la bioimpedancia bioeléctrica frente a la densitometría ósea en personas mayores de 55 años en función del sexo. XVI Reunión de la Sociedad Española de Nutrición. Octavas Jornadas de actualización de UNAV. Nutr Hosp 2013; 30 (supl 1):51-52. ISSN: 0212-1611. Pamplona (Spain). July, 2014.
 - Maroto-Sánchez B, Luzardo-Socorro R, Parylack SJ, **Aparicio-Ugarriza R**, Marín-Puyalto J, Palacios G, Tur JA, González-Gross M. Ingesta de líquidos en personas mayores. Diferencias entre personas físicamente activas y no activas.

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- **Aparicio-Ugarriza R.** Influence of physical activity on cell homeostasis and behavioural habit in Spanish adults over 55. II Jornadas de Jóvenes investigadores en nutrición. Pamplona (Spain). July, 2014.

Book chapters

- **Aparicio-Ugarriza R**, Galera J, Fuentes Jiménez F, González-Gross M. Aspectos básicos de la nutrición e hidratación deportiva. En Pleneufar 6: Educación Nutricional en la actividad física. ISBN: 978-84-7867-512-8.
- Gesteiro E, **Aparicio-Ugarriza R**, González-Gross M. Capítulo 6. Pautas en la alimentación del deportista y personas físicamente activas. Experto en Nutrición. Editorial Médica: Panamericana. ISBN: 978-84-9110-206-9.
- Cañada D, **Aparicio-Ugarriza R**, Calonge Pascual S, González-Gross M. 2nd Symposium for the exchange of experiences in promoting physical activity and health in schools. ISBN: 978-84-617-8378-6.

Organizations Scientific Events

- IX International Symposium in Strength Training. Organizing Committee. Universidad Politécnica de Madrid. Madrid (Spain). 16/12/2016-17/12/2017.
- Global Cancer, Occurrence, Causes, and Avenues to Prevention. 50th Anniversary International Agency for Research on Cancer. International Agency for Research on Cancer. Organizing Committee. Lyon (France). 07/06/2016 - 10/06/2016.
- 2nd Symposium for the exchange of experiences in promoting physical activity and health in schools. ImFINE Research Group. Universidad Politécnica de Madrid. Coordination, technical secretary and organization of the event. Madrid (Spain). 06/05/2016-07/05/2016.
- 1st Symposium for the exchange of experiences in promoting physical activity and health in schools. ImFINE Research Group. Universidad Politécnica de Madrid. Technical secretary and organization of the event. Madrid (Spain). 08/05/2015-09/05/2015.

- National Tournament of Boccia. Sports Federation of cerebral palsy. Organizing committee. Elche (Spain). 01/07/2011 - 03/07/2011.
- 6th International Congress of the Spanish Federation of the Physical Activity and Sports Sciences. Spanish Association of Spanish Association of Science in Physical Activity and Sport. Organizing committee. Elche (Spain). 05/10/2010-08/10/2010.

Project I+D+I

- Participation in Strategic Plan of Sustainability (Universidad Politécnica de Madrid). 30/06/2017.

Member of scientific societies

- Member of European College of Sport Science (ECSS).
- Member of Spanish Nutrition Society (SEÑ).
- Member of British Nutrition Society (BNS).
- Member of Spanish Research Network on physical activity and health (EXERNET).

Other achievements

- **Research member of consolidated Research Group:** ImFINE. Improvement (of health) by fitness, nutrition and exercise. Faculty of Physical Activity and Sport-INEF. Universidad Politécnica de Madrid. 2013-ongoing.
- **Peer reviewer of scientific articles:** 1) Clinical Chemistry and Laboratory Medicine; 2) British Journal of Nutrition; 3) European Journal of Nutrition; 4) Journal of Human Nutrition and Food Science; 5) Eating and Weight Disorders.
- The PhD Student has attended more than **40 courses**.