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Physical exercise, 1,25-dihydroxyvitamin D, and prevention of the consequences of ageing. The FIT-AGEING study

Ejercicio físico, niveles de 1,25-dihidroxivitamina D y prevención de las consecuencias del envejecimiento. Estudio FIT-AGEING

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*A mi familia y amigos, quienes, con
su cariño, ayuda y confianza me han permitido
seguir adelante y crecer en lo personal y profesional*

*A Sara, por apoyarme y permanecer
a mi lado durante todo el camino*

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RESEARCH PROJECTS AND FUNDING

ABBREVIATIONS

$1,25(OH)_2D$: 1,25-dihydroxyvitamin D

$24,25(OH)_2D$: 24,25-dihydroxy-cholecalciferol

$25(OH)_2D$: 25-hydroxyvitamin D

ALT: Alanine transaminase

ANCOVA: Analysis of covariance

ANOVA: Repeated-measures analyses of variance

ApoA1: Apolipoprotein ApoA1

ATP: Adenosine triphosphate

β: Standardized regression coefficient

BCa: Anthropometric and body composition assessment

β-cTX: Beta cross lap

BDNF: Brain-derived neurotrophic factor

BEDCA: Base de Datos Española de Composición de Alimentos

BMC: Bone mineral content

BMD: Bone mineral density

BMI: Body mass index

BSa: Blood samples assessment

CDV: Cardiovascular

CDVa: Cardiovascular disease risk assessment

CI: Confidence interval

CIDS: Centro de Investigación Deporte y Salud

CO_2: Carbon dioxide

COVID-19: Coronavirus disease-2019

CRP: C-reactive protein

CYP24: Enzyme 24-hydroxylase

CYP27B1: Enzyme 1-alpha-hydroxylase

DBP: Vitamin D binding protein

DIa: Dietary intake assessment

DXA: Dual-energy x-ray absorptiometry

ECG: Electrocardiogram

ENMO: Euclidean norm minus one

FDG: F-fluorodeoxyglucose

FGF21: Fibroblast growth factor-21

FGF23: Fibroblast growth factor-23

FGFR1c: Fibroblast growth factor receptor 1c

FINUT: Fundación Iberoamericana de Nutrición

FLI: Fatty liver index

FMI: Fat mass index

FOXO: Forkhead box protein

GABA: Gamma-aminobutyric acid

HDL-C: High density lipoprotein cholesterol

HIIT: High intensity internal training

HIIT+EMS: High intensity interval training adding whole-body electromyostimulation

HOMA-IR: Homoeostasis model assessment for insulin resistance index

HRres: Heart rate reserve

Hz: Hertz

IBD: Inflammatory Bowel Disease

IgG: Immunoglobulin G

IFG-I: Insuline-like growth factor -1

IFN-gamma: Interferon gamma

IL-2: Interleukin-2

IL-6: Interleukin-6

IU: International Units

ISAK: International Society for the Advancement of Kinanthropometry

LDL-C: Low density lipoprotein cholesterol

LMI: Lean mass index

LPA: Light physical activity

mA: Milliamps

MetScore: Cardiometabolic syndrome score

Min: Minutes

MPA: Moderate physical activity

MVPA: Moderate-vigorous physical activity

NCX: Sodium/calcium exchanger

Nrf2: NF-E2-related factor 2

O_2: Oxygen

OMS: Organización Mundial de la Salud

PA: Physical Activity

PAR: Concurrent training based on physical activity recommendation from the World Health Organization.

PAa: Physical activity assessment

PFa: Physical fitness assessment

PMCA: Plasma membrane calcium-ATPase

PTH: Parathyroid hormone

QUICKI: Quantitative insulin sensitivity check index

Reps: Repetitions

RM: Repetition maximum

RPE: Rating of perceived exertion

RXR: Retinoid X receptor

SE: Standar error

Sec: Seconds

S-Klotho: Shed form of the Klotho protein

SPSS: Statistical Package for Social Sciences

TNF-alpha: Tumor necrosis factor-α

TRPV6: Transient receptor potential vanilloid subfamily member 6

TRPV5: Transient receptor potential vanilloid subfamily member 5

TPRV-type: Transient receptor potential vanilloid type

Tr: Treadmill

USDA: U.S. Department of Agriculture

VDRE: Vitamin D response elements

VDRs: Vitamin D receptors

VO_2max: Maximum oxygen uptake

VPA: Vigorous physical activity

WB-EMS: Whole-body electromyostimulation

W-B: Weight-bearing exercises

WC: Waist circumference

WHO: World Health Organization

γ-GT: γ-glutamyl transferase

ABSTRACT

Ageing is a natural and multi-factorial process that affect human beings as they age, from born to death. At advanced age it is characterized by a progressive decline of physiological integrity leading to impaired physical and cognitive functions, and increasing the incidence of several age-related diseases. This late deteriorative phase of the ageing process can be named as aging, and the efforts related to prevent or delay the aging process as anti-aging. Recent studies have established the importance of vitamin D status on human' health during the ageing process beyond its role on specific physiological mechanisms in different organs and systems. In this sense, the main active metabolite of vitamin D, the 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), also known as calcitriol, is responsible for most of its biological effects, and considered a powerful biomarker that plays an important role in ageing-related physiological processes. However, little is known about their possible relationship with other ageing or, more precisely, aging biomarkers in healthy adult population. Furthermore, although previous studies have described that an exercise intervention induces important benefits for human health, the effects of physical exercise on $1,25(OH)_2D$ have not been deeply studied. On the other hand, there is no study comparing the influence of different physical exercise interventions on $1,25(OH)_2D$.

The main aims of this International Doctoral Thesis are to study the relationship of $1,25(OH)_2D$ and body composition, physical activity levels, physical fitness, cardiometabolic health, and the shed form of the Klotho protein (S-Klotho) (**Section 1**), and to study the effect of different exercise training programs on $1,25(OH)_2D$ (**Section 2**) in middle-aged sedentary adults. This International Doctoral Thesis includes a total of 5 studies.

The present results show that $1,25(OH)_2D$ was negatively associated with body mass index, lean mass index, and bone mineral density (**Study 1**), while objectively measured sedentary behaviour, physical activity levels, and physical fitness did not seem to be related to $1,25(OH)_2D$ (**Study 2**). Additionally, no association was found between $1,25(OH)_2D$ and cardiometabolic risk factors or insulin resistance, although an inverse association between $1,25(OH)_2D$ and central adiposity was observed (**Study 3**). On the other hand, $1,25(OH)_2D$ was negatively associated with S-Klotho plasma levels, which was partially mediated by bone mineral density (**Study 4**). Finally, a 12-weeks exercise program significantly increased the levels of $1,25(OH)_2D$ regardless of the exercise modality, observing a significant positive association between changes in physical fitness and changes in $1,25 (OH)_2D$ (**Study 5**).

Collectively, the results from the present International Doctoral Thesis enhance our understanding of the relationship of 1,25(OH)$_2$D with several anti-aging factors such as body composition, physical activity levels, physical fitness, cardiometabolic health, and S-Klotho protein in middle-aged sedentary adults. Moreover, it provides novel information regarding the role of different exercise training programs on 1,25(OH)$_2$D, as well as its potential relationship with exercise-induced changes in body composition and physical fitness.

RESUMEN

El envejecimiento es un proceso natural y multifactorial que se caracteriza por una disminución progresiva de la integridad fisiológica que conduce al deterioro de las funciones físicas y cognitivas y aumenta la incidencia de varias enfermedades relacionadas con la edad. Estudios recientes han establecido la importancia del estado de la vitamina D en la salud humana durante el proceso de envejecimiento más allá de su función sobre mecanismos fisiológicos específicos en diferentes órganos y sistemas. En este sentido, el principal metabolito activo de la vitamina D, la 1,25-dihidroxivitamina D ($1,25(OH)_2D$), también conocida como calcitriol, es responsable de la mayoría de sus efectos biológicos, y es considerada un poderoso biomarcador en diversos procesos fisiológicos relacionados con el envejecimiento. Sin embargo, existe excasa evidencia sobre su posible relación con otros biomarcadores de envejecimiento en la población adulta sana. Además, aunque estudios previos han descrito que una intervención de ejercicio induce importantes beneficios para la salud de los seres humanos, los efectos del ejercicio físico sobre la $1,25(OH)_2D$ no han sido estudiados en profundidad. De hecho, no existe ningún estudio que compare la influencia de diferentes intervenciones de ejercicio físico sobre la $1,25(OH)_2D$.

Los principales objetivos de la presente Tesis Doctoral Internacional son estudiar la relación de la $1,25(OH)_2D$ y la composición corporal, la condición física, la salud cardiometabólica y la proteína Klotho en su forma soluble (S-Klotho) (**Sección 1**), así como investigar el efecto de distintos programas de ejercicio físico sobre la $1,25(OH)_2D$ (**Sección 2**) en adultos sedentarios de mediana edad. Esta Tesis Doctoral Internacional incluye un total de 5 estudios.

Los resultados muestran que la $1,25(OH)_2D$ se asoció negativamente con el índice de masa corporal, el índice de masa magra y la densidad mineral ósea (**Estudio 1**), mientras que el comportamiento sedentario, los niveles de actividad física y la condición física (medidos objetivamente) no parecieron estar relacionados con la $1,25(OH)_2D$ (**Estudio 2**). Además, la $1,25(OH)_2D$ no se asoció con diversos factores de riesgo cardiometabólicos ni con la resistencia a la insulina, aunque se observó una asociación inversa entre la $1,25(OH)_2D$ y la adiposidad central (**Estudio 3**). Por otro lado, se puso de manifiesto una asociación negativa entre la $1,25(OH)_2D$ y los niveles plasmáticos de S-Klotho, siendo esta relación parcialmente mediada por la densidad mineral ósea (**Estudio 4**). Finalmente, un programa de ejercicio físico de 12 semanas de duración aumentó significativamente los niveles de $1,25(OH)_2D$ independientemente de la modalidad de ejercicio implementada, observándose una asociación positiva y significativa entre los cambios derivados de la intervención en la condición física y los cambios en los niveles de $1,25(OH)_2D$ (**Estudio 5**).

En conjunto, los resultados de la presente Tesis Doctoral Internacional proporcionan un avance en el conocimiento científico sobre la relación de la 1,25(OH)$_2$D con varios biomarcadores de anti-envejecimiento, tales como la composición corporal, los niveles de actividad física, la condición física, la salud cardiometabólica y la proteína S-Klotho en adultos sedentarios de mediana edad. Además, proporciona información novedosa sobre el papel que ejercen diferentes programas de ejercicio físico sobre la 1,25(OH)$_2$D, así como su potencial relación con los cambios inducidos por dicha intervención en la composición corporal y en la condición física.

GENERAL INTRODUCTION

Chapter 1

Physical exercise and nutrition: role in health and anti-aging effect

Ejercicio físico y nutrición: papel en la salud y efecto antienvejecimiento

INTRODUCCIÓN

No hay duda de que el estilo de vida ejerce una influencia directa en la salud, así como en la expectativa y la calidad de vida de los seres humanos [1]. Estilos de vida saludables se asocian con una menor incidencia de enfermedades, mayor nivel de bienestar, mejor calidad de vida y más prolongada [2,3]. Por el contrario, estilos de vida poco saludables tienen consecuencias negativas sobre la salud y dan lugar, antes o después, a enfermedades crónicas y degenerativas, muchas de ellas asociadas también al propio proceso de envejecimiento [2,3]. El ejercicio físico y la alimentación son factores fundamentales del estilo de vida y constituyen las mejores herramientas hoy disponibles para mejorar la salud y luchar contra las inevitables consecuencias del envejecimiento, determinando así una longevidad saludable [4–9].

SALUD

La salud, tal como la definió la Organización de la Salud (OMS) en 1946, es "el estado de completo bienestar, físico, mental y social, y no sólo la ausencia de enfermedad" [10]. Pero la salud no es un estado estable, sino más bien un status quo: el estado existente en un determinado momento, sometido a un difícil equilibrio susceptible de cambiar, generalmente para mal. Uno puede gozar de buena salud, incluso excelente, tener un accidente y perder, en mayor o menor medida, esa buena salud. Se puede estar bien, pero recibir una mala noticia, comer algo indigesto o dormir mal, e inmediatamente perder salud, poca o mucha. Salud que a veces se consigue recuperar y otras veces, desgraciadamente, no. Muchos factores afectan o pueden afectar negativamente ese status quo, y muy pocos pueden mejorarlo. Y cuando la mejora ocurre es, sobre todo, cuando se ha perdido salud, generalmente de forma aguda, y el objetivo (que es el objetivo de la medicina) es recuperar cuanto antes esa salud perdida. Es decir, intentar volver al status quo previo. Unas veces la pérdida de salud ocurre de manera abrupta. Otras veces, la pérdida es progresiva y paulatina. La recuperación de la salud, cuando ocurre, suele ser un proceso lento. Cuando ya se parte de una buena situación, de un buen nivel de salud, el proceso de ganancia de salud ocurre de manera especialmente lenta. Además, el nivel de salud que en un momento se tiene es un punto específico dentro de un continuum extremadamente amplio, tan amplio como la vida humana. Si pudiera cuantificarse el nivel de salud entre el mínimo posible (la persona que va a morir en el instante siguiente) y el que no puede ser mejor (la que se encuentra en su mejor estado posible de forma, física, mental, social), en un punto u otro, en cualquier momento, estamos todos entre medio [11].

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ENVEJECIMIENTO Y ANTIENVEJECIMIENTO

Evidentemente, el envejecimiento no es una enfermedad, pero tiene muchas características que podrían llevar a considerarla como tal [12]. En condiciones normales, el envejecimiento fisiológico se acompaña, a partir de la década de los 30 años o incluso antes, de un declive funcional a distintos niveles [13]. Esto determina las manifestaciones clínicas propias del paso de los años y se asocia también al desarrollo de las diversas enfermedades asociadas al envejecimiento [14]. Como tal, el envejecimiento es incurable, por definición mortal y su incidencia es universal. Por lo tanto, existen argumentos para considerarlo como una enfermedad crónica susceptible de ser diagnosticada para determinar su nivel de evolución, también de ser tratada para enlentecer (no para detener) su progresión y también, asociado a lo anterior o no, de ser prevenida [14,15].

Dado que, en la actualidad, como consecuencia del mejor control de las enfermedades infecciosas y agudas, cada vez se vive más tiempo, el envejecimiento como problema de salud afecta a un porcentaje cada vez mayor de la población y puede denominarse como la enfermedad del siglo XXI, lo que implica la necesidad de abordar el problema con distintas estrategias que consigan disminuir sus efectos y atenuar sus consecuencias [16]. En este contexto, podría hablarse de envejecer mejor, con más éxito (successful aging), aunque un término que en este sentido ha ganado enorme popularidad y se encuentra admitido es el de antienvejecimiento [17]. En realidad, este vocablo no hace referencia a detener el envejecimiento sino a ralentizar el declive funcional que lleva asociado y a prevenir las enfermedades relacionadas con él [17]. Por consiguiente, el objetivo no sería tanto añadir años a la vida, como añadir vida a los años, es decir, no prolongar la vida sino tener una vida activa y plena el mayor tiempo posible [18].

Como se ha mencionado, el envejecimiento, aunque inevitable, es influenciable. Se puede acelerar (y, de esta forma, envejecer más rápido) o enlentecer (y, de esta forma, envejecer más lento) [14,17]. Y ello con independencia de las características de la persona. Envejecer más lento significa envejecer menos, resistir mejor el paso del tiempo y eso es, literalmente, antienvejecimiento [18].

Asimismo, para alguna estructura o función concreta podría virtualmente detenerse el proceso de envejecimiento. Imagínese, por ejemplo, reemplazar una estructura orgánica por otra estructura artificial que sea prácticamente inmune al paso del tiempo y sus consecuencias (p. ej., el cristalino). Para esa estructura y para esa función el paso del tiempo, el envejecimiento, y sus consecuencias (tal como lo entendemos desde un punto de vista fisiológico) se habrían detenido [19]. Eso sería también antienvejecimiento. Es decir, para esa función concreta no se envejece.

Por otra parte, con respecto a alguna estructura o función concreta es posible conseguir mejorar sus características estructurales y su capacidad funcional llevándolas a un nivel con el que aparenten tener menos edad o realmente se consiga recuperar una capacidad funcional o adaptativa que ya se había perdido o que incluso no se había llegado a tener previamente. Por ejemplo, la aparición de una arruga de expresión es una consecuencia del envejecimiento, pero si se trata, por ejemplo, con toxina botulínica y desaparece temporalmente dotando a la persona de un aspecto similar al que tenía con menos edad, se habrá revertido el envejecimiento [20]. O la fuerza, por ejemplo, que se va perdiendo con el paso de los años pero que con un programa de entrenamiento adecuado se puede recuperar al nivel que se tenía previamente, cuando se era más joven, o incluso alcanzar un nivel de fuerza o habilidad que nunca se había tenido antes [21,22]. Todo esto es, literalmente, también, antienvejecimiento. Es decir, se ha revertido el envejecimiento o su consecuencia.

Por último, con la edad, debido al deterioro estructural y a la pérdida de capacidad funcional, se produce también una serie de afecciones o enfermedades ligadas a la edad, muchas de las cuales son de carácter degenerativo [23,24]. Su gravedad y su repercusión para la salud son muy variables. Algunas apenas tienen repercusión, por ejemplo, la calvicie [25]. En otras, las consecuencias son relativamente importantes, como es la pérdida progresiva de audición o de visión [26]. Otras alteraciones son muy progresivas y sólo cuando ya el proceso está muy avanzado se diagnostican, pasando a ser enfermedades graves como es el caso de la insuficiencia renal o la pérdida de capacidad cardiorrespiratoria y de movilidad o autonomía [27–29].

No obstante, las enfermedades que más llaman la atención y requieren también más cuidado son las más graves, las que confieren más pérdida de autonomía y mayor coste social y sanitario, así como también mayor mortalidad [23]. Entre ellas destacan las enfermedades neurodegenerativas, siendo uno de sus principales exponentes la enfermedad de Alzheimer [30], pero también la aterosclerosis [31], las enfermedades cardiovasculares [32], enfermedades metabólicas como la diabetes de tipo 2 [33] o diversas formas de cáncer [34]. Muchas de estas enfermedades tienen, e incluso comparten, diversos factores de riesgo, como son, por ejemplo, el tabaquismo [35], la falta de ejercicio [36] o una alimentación inadecuada [37]. Luchar contra estos factores de riesgo e intentar controlarlos, prevenir el desarrollo de dichas enfermedades degenerativas, atenuar o reducir sus consecuencias e incluso contribuir a su tratamiento serían también medidas antienvejecimiento.

Para conseguir este objetivo, es fundamental crear y adoptar estrategias que tengan como objetivo ralentizar el proceso de envejecimiento [38–40]. Entre esas estrategias hoy se acepta como ineludible el hecho de mantenerse física y mentalmente activo, así como ser autosuficiente y estar socialmente integrado [17,41] En todo ello, la práctica regular de ejercicio físico cobra un papel

fundamental. Pero ¿qué ejercicio?, ¿con qué intensidad?, ¿cuál es el volumen o el tiempo de ejercicio preciso o mínimo imprescindible para obtener los efectos deseados? Hace ya tiempo se planteó que la respuesta a estas preguntas podía resumirse en un concepto: el tipo de ejercicio practicado con la intensidad y durante el tiempo necesario para conseguir mantener o incluso intentar aumentar o mejorar la condición física. Muchos son los resultados de las investigaciones que se han publicado en relación con este tema desde entonces y todos van en la misma línea [21,42,43].

EJERCICIO FÍSICO COMO MEDICINA

La práctica regular de actividad física y el mantenimiento de un buen estado de forma física son de capital importancia para la salud y el bienestar de la persona [8,9,40]. Cuando se realiza de manera adecuada, los beneficios del ejercicio se producen siempre, con independencia de la edad, el estado de salud y la condición física de la persona. La evidencia científica disponible así lo ha puesto de manifiesto de manera inequívoca [42,44-47]. El ejercicio físico practicado de manera regular y en la forma adecuada ha demostrado ser un medio efectivo para reducir la morbimortalidad ocasionada por numerosas y frecuentes enfermedades crónicas [44,48-51]. Por otro lado, también se ha demostrado que las personas físicamente activas viven más y mejor (con mayor calidad de vida) que las personas sedentarias [52,53]. Por último, las personas físicamente activas se encuentran personal y socialmente mejor [54]. Dada esa multiplicidad de efectos beneficiosos para la salud y el bienestar de las personas, los principales organismos de salud de los países desarrollados han puesto en marcha campañas enérgicas destinadas a fomentar la actividad física entre los ciudadanos. El propio National Institute of Health norteamericano ha considerado el ejercicio físico como la principal «píldora» para luchar contra las consecuencias del envejecimiento [55]. En esta misma línea, el Departamento de Salud Norteamericano sitúa la actividad física como el primero de los diez indicadores de salud, por delante del sobrepeso/obesidad o el tabaco, y lo considera el principal elemento de acción de su agenda de trabajo para los próximos años [55].

A pesar del indudable beneficio que aporta la práctica de ejercicio, la mayoría de las personas, tanto jóvenes como adultos o ancianos, llevan una vida que puede considerarse sedentaria [56,57]. Este problema se va acentuando con el paso de los años y tiene consecuencias negativas no sólo para la persona sino también para la familia y la sociedad, dada la sobrecarga y el coste económico que determinan las enfermedades ligadas al sedentarismo y sus graves consecuencias [58,59]. Puede decirse que la falta de ejercicio acelera el envejecimiento y sus consecuencias, una de las cuales es el propio aspecto de la persona. Entre personas de la misma edad y el mismo sustrato genético, las que se mantienen físicamente activas, se alimentan de

manera saludable y evitan la exposición a factores de riesgo presentan un aspecto más joven y saludable [60].

EJERCICIO EN EL PROCESO DE ENVEJECIMIENTO

La práctica de ejercicio de manera moderada durante al menos 30 minutos, 5 días a la semana, o de ejercicio intenso durante 20 minutos 3 días por semana es fundamental para mantener y mejorar la salud con el paso de los años [21]. De forma sencilla y fácil de recordar, puede hablarse de la conveniencia de realizar 59 minutos al día de actividad física intencionada, la mayor parte de los días de la semana. Un número, 59, que pone de manifiesto la importancia de llegar, pero también la importancia de no pasarse. La práctica de ejercicio tiene efectos beneficiosos en la mayoría, si no en todas, las funciones orgánicas, contribuyendo a mantener su funcionalidad e incluso a mejorarla [6,61]. Dado que la pérdida de funcionalidad que se produce con la edad es, precisamente, la principal consecuencia del envejecimiento, el efecto del ejercicio puede considerarse una verdadera terapia para luchar contra las inevitables consecuencias del proceso de envejecimiento [9,42,61].

En la mayoría de los casos, el ejercicio actúa retardando o atenuando la pérdida de funcionalidad. Para algunas funcionalidades el ejercicio actúa, incluso, revirtiendo una pérdida ya establecida [6,40]. De manera específica y directa, el ejercicio físico mantiene y mejora las funciones musculoesquelética [21], osteoarticular [62,63], cardiocirculatoria [46], respiratoria [21], endocrinometabólica [64], inmunitaria [65] y psiconeurológica [66,67]. Realizar ejercicio físico de manera regular reduce el riesgo de desarrollar o incluso morir de lo que hoy día son las principales y más graves causas de morbimortalidad en los países occidentales [36,51,68]. Así, el ejercicio físico practicado de manera regular y de forma adecuada es un determinante de la mejora de la salud y el bienestar de la persona dado que:

- Reduce el riesgo de cardiopatía isquémica y otras enfermedades cardiovasculares.
- Disminuye el riego de desarrollar obesidad y diabetes.
- Reduce el riesgo de desarrollar hipertensión o dislipidemia y ayuda a controlarlas.
- Limita el riesgo de desarrollar cáncer de colon y de mama.
- Ayuda a controlar el peso y la imagen corporal.
- Tonifica los músculos y preserva o incrementa la masa muscular.
- Vuelve los huesos y las articulaciones más fuertes y resistentes.
- Aumenta la capacidad de coordinación y respuesta disminuyendo el riesgo y las consecuencias de las caídas.

- Mejora la actividad del sistema inmunitario.

- Reduce los sentimientos de depresión y ansiedad.

- Promueve el sentimiento psicológico de bienestar y la integración social.

Estas acciones se presentan a cualquier edad y con independencia del nivel de forma física de origen. En este sentido es preciso distinguir dos conceptos que, aunque interrelacionados y mutuamente influenciables, son claramente diferentes. Estos conceptos son los de forma física (o condición física) y actividad física [69,70]. Los términos actividad física, ejercicio físico, deporte y condición física (physical fitness) suelen utilizarse de forma confusa; aunque son términos estrechamente relacionados, no deben utilizarse como sinónimos. La actividad física hace referencia a cualquier movimiento corporal producido por el músculo esquelético que se asocia a consumo energético [70]. La actividad física puede ser la que se realiza en los quehaceres de la vida diaria (actividad física de la vida diaria), en el trabajo (actividad física laboral) o en el tiempo de ocio. Esta última tiene la característica de que se realiza de manera voluntaria y como alternativa a otro tipo de actividades que pueden ser sedentarias. El concepto de actividades sedentarias es interesante y no es contrapuesto al de actividad física. Actividades sedentarias son aquellas que se realizan estando sentado, reclinado o de pie sin moverse o moviéndose poco [69]. No es un concepto contrapuesto al de actividad física, porque una persona puede ser físicamente activa, si practica ejercicio, y ser también sedentaria si se pasa muchas horas sentado, por ejemplo, trabajando. El sedentarismo es un factor de riesgo para diversas enfermedades asociadas al proceso de envejecimiento [71].

El ejercicio físico se define como la actividad física planificada, estructurada, sistemática y realizada por placer o dirigida a la mejora o el mantenimiento de uno o más componentes de la condición física, siendo conscientes de ello o sin serlo [70]. Puede definirse como ejercicio físico invisible una forma de actividad física que reúne todas las características de ejercicio físico, pero sin que aparentemente se esté realizando [8]. Sería el caso de sistemáticamente no utilizar ascensores, ni escaleras o pasarelas automáticas, desplazarse andando en lugar de recurrir al coche o transporte público para desplazamientos cortos, aparcar en sitios más lejanos, levantar objetos en vez de arrastrarlos, etc. Todas estas actividades practicadas de forma planificada, estructurada y sistemática con la finalidad de realizar actividad física son una forma de ejercicio que puede pasar inadvertida, de ahí el nombre de ejercicio invisible [8]. El deporte es una forma de ejercicio físico que se practica ateniéndose a unas reglas, las reglas del propio deporte [72]. El juego sería una forma de actividad deportiva en la que las reglas cambian al gusto o necesidad de quien lo realiza [72].

CONDICIÓN FÍSICA COMO MARCADOR DE SALUD Y FACTOR ANTIENVEJECIMIENTO

La condición física es la medida de la capacidad que se tiene para realizar actividad física y/o ejercicio físico integrando, para ello, la mayoría de las estructuras y funciones corporales involucradas en el movimiento corporal, como sistema/función locomotora, cardiorrespiratoria, hematocirculatoria, endocrinometabólica, psiconeurológica, etc. [42]. La condición física integra diversos componentes, entre los que destacan la condición cardiorrespiratoria o capacidad aerobia, la fuerza muscular, factores neuromusculares y otros asociados, entre los que se incluyen la coordinación, el equilibrio estático y dinámico, la flexibilidad, la postura y el tiempo de reacción [73]. Por último, puede considerarse, como hace la OMS, la composición corporal como el cuarto componente de la condición física (Figura 1) [73].

Figura 1. Componentes de la condición física que influyen en el proceso de envejecimiento

Puede decirse que la condición física constituye una medida integrada de todas las funciones y estructuras que intervienen en la realización de una actividad física o ejercicio [42]. Es decir, representa una medida de la capacidad de que se dispone para practicarla. Con la edad, esta capacidad va sufriendo un declive progresivo, que en promedio se estima ocurre a razón de un 10 % por década [13]. Al principio, para la mayoría de las personas, esa pérdida de capacidad pasa bastante inadvertida, porque la reserva funcional de que se dispone es importante y en casi todas las ocasiones uno no se exige la realización de un esfuerzo máximo. Sólo en los deportistas de élite que cuando compiten (y a veces también cuando entrenan) se autoexigen ese esfuerzo máximo, se pone de manifiesto esa mengua de capacidad, esa pérdida de condición física, de una forma temprana. Para las demás personas, la pérdida de estado de forma sólo suele hacerse

aparente cuando ya no se pueden llevar a cabo actividades comunes que previamente se realizaban sin problema (subir unas escaleras o una cuesta, levantar un peso, etc.) [8].

Si se estima que la capacidad funcional es máxima en la década de los 20 años y que las manifestaciones clínicas de insuficiencia funcional se producen cuando se ha agotado el 80 % de capacidad funcional (que es lo que corresponde a la reserva funcional), puede estimarse que en condiciones ideales, en ausencia de otros factores que influyan negativamente en el proceso, o sea que aceleren la pérdida, como enfermedades o traumatismos, podría mantenerse un excelente nivel de salud, permaneciendo sin enfermedades asociadas al envejecimiento, hasta los 100 años [13]. A partir de ese momento se produciría el fenómeno conocido como compresión de la morbilidad, es decir, aparecerían de manera acumulada manifestaciones de insuficiencia en los diversos órganos y sistemas, fueran éstos vitales o no [74,75]. En dos décadas más, o sea en torno a los 120 años, el agotamiento de la capacidad funcional sería generalizado, y la afectación de un órgano vital determinaría la muerte del individuo. Esto coincide con la máxima longevidad de la que hay registros históricos fiables y que presenta como techo los 122 años, sólo alcanzados por la francesa Jeanne Calment, la única persona que ha superado los 120 años de edad, aunque hay otras muchas personas que han conseguido acercarse a este techo [76]. Conseguir enlentecer la velocidad de pérdida de capacidad funcional, por ejemplo, al 8-9 % sería una verdadera y efectiva terapia antienvejecimiento, no ya por lograr vivir más sino por mantener la buena salud y la capacidad funcionar para hacer frente a las actividades de la vida diaria durante más años, idealmente hasta los 90-100 años de edad, algo que sí es socialmente evidente que consiguen muchas personas, ya que cada vez son más los centenarios sanos [77]. En cuanto a la condición física como marcador integrado de dicha capacidad, esto resulta, a priori, perfectamente factible mediante un adecuado programa de entrenamiento que consiga, al menos, preservarla o enlentecer su declive [78].

Recientes investigaciones han profundizado acerca de la importancia de conocer el nivel de condición física de una persona como método para valorar su capacidad funcional, estado de salud, expectativa y calidad de vida, resultando ser este parámetro en la actualidad el mejor factor predictivo de mortalidad por todas las causas [78]. Resulta, pues, fundamental controlar el nivel de condición física durante el proceso de envejecimiento a través de su evaluación, con objeto de demostrar su declive, plantear estrategias para mantenerla o mejorarla y adecuar las exigencias físicas a las posibilidades reales de cada individuo mediante programas individualizados de entrenamiento [8].

CAPACIDAD AERÓBICA COMO ÍNDICE DE SALUD

La condición física cardiorrespiratoria o capacidad aeróbica constituye uno de los pilares de la condición física de un individuo, y el consumo máximo de oxígeno (VO$_2$máx), la variable fisiológica que mejor la define [79,80]. Puede expresarse en términos absolutos de consumo de oxígeno (l/min) cuando se realiza un esfuerzo máximo, en términos relativos (ml/kg/min) o en equivalentes metabólicos (en múltiplos MET, siendo 1 MET = 3,5 ml/kg/min, que es aproximadamente la energía que una persona sana consume en condiciones basales o de reposo).

El VO$_2$máx puede estimarse de manera directa, a través de una prueba de esfuerzo máxima o submáxima, generalmente andando o corriendo en un tapiz rodante o pedaleando en un cicloergómetro [81]. Simultáneamente, se mide el oxígeno consumido y el dióxido de carbono producido mediante un analizador de gases. También es posible estimar la intensidad del esfuerzo y, a partir de ahí, de manera indirecta, el consumo de oxígeno, mediante el registro de la frecuencia cardíaca [82,83]. En este caso pueden utilizarse diversas pruebas tanto de laboratorio como de campo, ya que el registro de la frecuencia cardíaca es mucho más sencillo.

Las conclusiones obtenidas en estudios clásicos respecto a la capacidad aeróbica y la morbimortalidad han sido confirmadas por estudios publicados recientemente mejor controlados, con mayor número de participantes y mayor tiempo de seguimiento. Estos estudios ponen claramente de manifiesto que el VO$_2$máx es un potente factor predictivo de mortalidad por todas las causas (especialmente por enfermedad cardiovascular), tanto en hombres como en mujeres sanos de diferentes edades [84,85], y además añaden que esa correlación es independiente de factores como el consumo de alcohol o tabaco y el síndrome metabólico [44,86,87]

Existen diversas investigaciones con respecto a la capacidad aeróbica y el cáncer, las cuales establecen relaciones entre ambos. Una revisión publicada recientemente pone de manifiesto que las personas con alta capacidad aeróbica tienen un 45 % menos de riesgo de mortalidad total por cáncer de cualquier tipo en comparación con las que poseen un bajo nivel de dicha capacidad, con independencia del porcentaje de grasa que la persona posea [88]. Se ha descrito la existencia de una asociación inversa entre la mortalidad por cáncer (en este caso de pulmón y/o colorrectal) y una alta capacidad aeróbica en adultos de mediana edad [89].

En relación con la función cognitiva y la salud mental, existe evidencia clara acerca de la asociación entre capacidad aeróbica y deterioro cognitivo [90]. Un estudio publicado recientemente pone de manifiesto que niveles altos de capacidad aeróbica en etapas iniciales y medias de la vida parecen estar asociados con un riesgo más bajo de desarrollo de demencia senil con el paso de los años, con independencia del padecimiento o no de enfermedad cerebrovascular [91]. Asimismo,

otro estudio reciente sugiere que existe una relación inversa entre la capacidad cardiorrespiratoria y el riesgo de mortalidad por demencia senil [92]. Por otro lado, si se tienen en cuenta las variables función y rendimiento cognitivo y se relacionan con la capacidad aeróbica, diversos estudios aportan evidencias científicas sobre la relación entre altos niveles de ésta y mejores memoria verbal y velocidad psicomotora [93]; además, existe relación entre altos niveles de condición cardiorrespiratoria y menor pérdida de la función ejecutiva y la memoria episódica en adultos mayores de 69 años [94].

Por último, en relación con factores socioeconómicos de la población, se ha publicado recientemente un artículo científico que concluye que niveles adecuados de condición cardiorrespiratoria en la edad adulta están fuertemente asociados con costes más bajos en cuanto a atención de salud en un promedio de 22 años más tarde en la vida, con independencia de los factores de riesgo cardiovasculares [95].

FUERZA COMO FACTOR PREVENTIVO DE MORBIMORTALIDAD Y ENVEJECIMIENTO

La fuerza es un factor que ha sido, y es en la actualidad, objeto creciente de estudio por parte de expertos en el proceso de envejecimiento. Trabajos recientemente publicados han sugerido la existencia de una asociación inversa entre la fuerza muscular y el riesgo de mortalidad [96]; dichas conclusiones han sido confirmadas en una revisión que puso de manifiesto la relación descrita anteriormente con independencia de cualquier causa o factor, como edad [97], grasa corporal, tabaquismo, hipertensión [98] o consumo de alcohol [99] e, incluso, sin considerar la propia capacidad aerobia [99].

El proceso de envejecimiento está asociado a una pérdida lenta pero inexorable de masa muscular, fuerza y capacidad funcional (sarcopenia y dinapenia); por lo tanto, el mantenimiento y la mejora de estos parámetros deben ser un hecho de consideración como método de prevención de enfermedad asociada al proceso de envejecimiento [100,101].

Los mecanismos y razones por los cuales se explica el efecto de la cualidad física fuerza o aptitud neuromuscular sobre la morbimortalidad son múltiples. De algún modo, el nivel de fuerza refleja y se relaciona con la masa muscular [102] y/o con el nivel de actividad física [100], por lo que, a mayor pérdida de fuerza, más acelerado el proceso de envejecimiento y, en consecuencia, mayor incidencia de enfermedades asociadas y mayor riesgo de mortalidad por cualquier causa [101].

La sarcopenia es un síndrome que se caracteriza por una pérdida gradual y generalizada de la masa muscular esquelética y, por lo tanto, de la fuerza, con riesgo de provocar limitaciones funcionales y consecuencias adversas, entre ellas dificultad laboral o en las actividades de la vida diaria, discapacidad física, calidad de vida deficiente y mortalidad, en este caso ocasionada principalmente por el propio proceso de envejecimiento [103]. Está bien documentado que el mantenimiento de la fuerza a partir de su entrenamiento puede contrarrestar el declive natural de la masa muscular a través de la generación de hipertrofia y fuerza muscular para así paliar el proceso de sarcopenia [104].

Otro factor influyente en la aceleración del envejecimiento es la osteoporosis, proceso en el que ocurre una pérdida progresiva de la densidad mineral ósea que tiene como consecuencia un aumento de la fragilidad ósea [105]. Este proceso es muy acusado, de forma característica, en mujeres posmenopáusicas [106], lo que clásicamente ha condicionado la mayor incidencia de fracturas de cadera en el sexo femenino [107]. Sin embargo, durante los últimos, el número de fracturas óseas ocasionadas por este fenómeno ha aumentado de forma significativa entre los hombres [108]. Los resultados de una investigación reciente muestran una relación positiva entre las fracturas de cadera en individuos con osteoporosis y el riesgo de morbimortalidad por cualquier causa, siendo éste, por lo tanto, un factor que contribuye al envejecimiento prematuro [109].

Asimismo, en recientes estudios se ha constatado que existe una relación positiva entre la densidad mineral ósea (indicador de osteoporosis) y la fuerza muscular tanto en valores de fuerza de prensión manual como en potencia de extensores de rodilla [110]; por lo tanto, cabe destacar la importancia del mantenimiento y/o el incremento de la cualidad física fuerza en el proceso de osteopenia, con el fin de mantener la capacidad funcional del individuo y conseguir una disminución del riesgo de sufrir caídas y fracturas óseas y, con ello, ser utilizada como terapia antienvejecimiento [111].

Por otro lado, en relación con la función endocrina del músculo, diversos estudios actuales han investigado el papel de las miocinas en la contracción muscular [112]. La célula muscular esquelética, a través de la secreción de miocinas, cumple funciones como la regulación metabólica y el control de la inflamación crónica asociada [113]. Dichas miocinas aumentan la lipólisis y mejoran la sensibilidad a la insulina, como las interleucinas (IL) 6 y 15, el factor neurotrófico derivado del cerebro (BDNF), el factor de crecimiento fibroblástico 21 (FGF-21), y la visfatina y otras hormonas a través de su acción paracrina participan en la adaptación muscular, como la IL-8, LIF y el FGF-21; conociendo que las alteraciones de los procesos de regulación metabólica y de inflamación forman parte del proceso patogénico que lleva al desarrollo de la obesidad y, consecuentemente, a la resistencia a la insulina, así como está implicado en la

patogenia de la aterosclerosis, la neurodegeneración y el crecimiento tumoral, se revela como altamente conveniente la adecuada producción de dichas miocinas [114,115].

Por otra parte, dado que el músculo esquelético es el principal receptor y modulador de la acción de la insulina, la pérdida de tejido muscular (que se asocia a la pérdida de fuerza) puede predisponer al desarrollo de determinadas enfermedades metabólicas, como resistencia a la insulina, diabetes mellitus de tipo 2 o síndrome metabólico [101,102], y en última instancia estar relacionado con la mortalidad prematura y el proceso de envejecimiento [99] (Tabla 1).

Tabla 1. Asociación entre fuerza muscular y morbimortalidad

- Mejora de los factores de riesgo cardiovascular
- Reducción de la resistencia a la insulina
- Mejora de la función y la calidad musculares
- Incremento del metabolismo basal
- Reducción del riesgo de caídas
- Prevención de la pérdida ósea con la edad
- Reducción de la inflamación sistémica
- Mejora de la función cognitiva

Por lo tanto, puede concluirse que la fuerza muscular tiene un papel fundamental en la prevención de enfermedades relacionándose muchas de ellas con el proceso de envejecimiento.

COMPOSICIÓN CORPORAL Y ENVEJECIMIENTO

La composición corporal, entendida al menos como la cantidad, la proporción y la distribución de masa grasa y masa magra (y, dentro de ella, masa muscular), se considera un importante indicador de salud asociado también al desarrollo o la prevención de diversas enfermedades y al mantenimiento, o no, de la salud a lo largo de los años [42]. Estudios anteriores ya hacían referencia a los cambios producidos en la composición corporal durante el proceso de envejecimiento, específicamente el aumento del tejido adiposo o porcentaje de masa grasa corporal y la disminución tanto de la cantidad como de la calidad del tejido muscular esquelético y/o decremento del porcentaje de masa muscular [116].

En la población de la mayoría de los países desarrollados, y en un creciente número de habitantes de países en vías de desarrollo, el aumento de la adiposidad es una evidencia incontrovertible; dicho aumento incrementa no sólo la tasa de personas con sobrepeso sino también la de personas con obesidad, la cual, según diversos estudios, se considera la principal

causa de discapacidad en adultos mayores [117]. Se estima que el 37 % de los hombres y el 42 % de las mujeres mayores de 60 años de edad son obesos, considerando el índice de masa corporal (IMC) como indicativo de obesidad y siendo este mayor o igual a 30 kg/m² [118]. Estos porcentajes son especialmente preocupantes; así, según un meta-análisis reciente que estudió la relación existente entre el IMC y la disminución de la capacidad funcional en mayores, la ocurrencia de dicha pérdida de capacidad es un 60 % más probable en personas mayores que presentan un IMC superior a 30 kg/m² [119]. En relación con el tejido adiposo y el porcentaje de masa grasa corporal, existen diversos estudios actualizados, llevados a cabo en población sénior, que relacionan ésta con un mayor riesgo de padecer enfermedades crónicas, como enfermedad cardiovascular, diabetes y/o cáncer [120], lo que contribuye indirectamente a la disminución de la capacidad funcional y acelera el proceso de envejecimiento [121,122]. Limitar el aumento progresivo de ganancia de grasa que ocurre con los años y disminuir el porcentaje de grasa mediante aumento de la actividad física son, en sí mismos, una verdadera intervención antienvejecimiento.

El tejido adiposo puede considerarse un órgano que, a diferencia de otros, se encuentra distribuido por amplias zonas del cuerpo. Diversos estudios ponen de manifiesto que no sólo es importante la cantidad de tejido adiposo, sino también el tipo de distribución de la grasa [123]. La adiposidad central (obesidad androide), especialmente la visceral, se considera la más peligrosa en relación con la proliferación de enfermedades cardiovasculares en comparación con la distribución mayoritaria de tejido adiposo en zonas más periféricas u obesidad ginecoide [124].

En cuanto a la pérdida de masa muscular que ocurre con el paso de los años (sarcopenia), es preciso añadir que la pérdida de fuerza es bastante más rápida que la pérdida concomitante de masa muscular, lo que sugiere una disminución significativa en la calidad muscular debido fundamentalmente a factores neuromusculares; por lo tanto, resulta fundamental indagar más sobre estrategias que aumenten la calidad muscular durante el proceso de envejecimiento, además de mantener o aumentar la masa muscular para prevenir o retardar el deterioro funcional, tanto en hombres como en mujeres de edad avanzada, como método antienvejecimiento [125].

La adiposidad y la sarcopenia, por lo tanto, son dos factores que contribuyen de manera independiente a la disminución de la capacidad funcional y autonomía personal en personas mayores, así como a la aceleración del proceso de envejecimiento, pero los efectos sinérgicos de estos cambios en la composición corporal, es decir, la variación relativa de uno y otro, agravan aún más los anteriores procesos [126].

Si se separan ambos conceptos, un estudio publicado recientemente sugiere que la obesidad (sin sarcopenia) es un factor de riesgo mayor que la sarcopenia (sin obesidad) en

relación con la pérdida de funcionalidad en población sénior; sin embargo, otro estudio actual pone de manifiesto que la combinación de ambos se considera una enfermedad conocida con el nombre de obesidad sarcopénica, cuya presencia implica un decremento mayor en la capacidad funcional y en el riesgo de padecer arteriosclerosis, diabetes de tipo 2 o síndrome metabólico [127].

ALIMENTACIÓN, NUTRICIÓN Y DIETA

La alimentación equilibrada forma parte esencial de un estilo de vida saludable [128,129]. Desde el punto de vista metabólico, la vida humana se mantiene al garantizarse un adecuado balance entre lo que el organismo consume en el mantenimiento de sus funciones vitales y lo que se provee mediante el complejo proceso de la nutrición, por el cual, el organismo recibe, transforma y utiliza los nutrientes contenidos en los alimentos [130]. El proceso a través del cual el organismo se provee voluntariamente de alimentos es lo que se denomina alimentación. Lo que el organismo hace con los alimentos, la forma en que se nutre, es lo que se conoce como nutrición [130]. Si la alimentación, y por lo tanto la nutrición, tiene una finalidad terapéutica o persigue un fin (p. ej., antienvejecimiento o mejora del rendimiento) entonces se habla de dieta [131].

La alimentación se produce de forma intermitente, mientras que el organismo necesita disponer de nutrientes de manera continua, pero, además, en cantidad y calidad variables, según las circunstancias metabólicas, ya sea en el corto plazo, en el medio, o en el largo plazo (como es el envejecimiento) [132]. En consecuencia, se ha de disponer de mecanismos adecuados para poder hacer frente a las cambiantes necesidades metabólicas, existiendo para ello una serie de hormonas encargadas de ajustar de manera específica tal adecuación [133]. Estos sistemas son, sin embargo, incapaces de hacer frente a situaciones mantenidas de desequilibrio metabólico, como ingestas alimentarias globales o específicas que difieren de las necesidades nutricionales, bien por exceso, bien por defecto. Cuando éste es el caso, se originan afecciones metabólicas de gran incidencia y/o gravedad, como son, por un lado, obesidad, dislipemias, aterosclerosis, diabetes, hipertensión arterial y, por otro lado, desnutrición, anemia, déficit vitamínico, etc. [37,134,135]. Estas situaciones son particularmente graves cuando afectan a personas de riesgo o genéticamente predispuestas.

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Chapter 2

Vitamin D/ Calcidiol/ Calcitriol: Role in ageing and anti-aging

INTRODUCTION

We shall begin this review by making a terminological clarification regarding the different substances that are usually grouped under the term Vitamin D. In addition, each of them can be named in different ways. Vitamin D itself is cholecalciferol (also named vitamin D3), which sense strict is not a vitamin since it is mainly endogenously synthesized, specifically in the skin with the help of ultraviolet radiation from sunlight. Cholecalciferol is an inactive substance from a biological point of view. An alternative source of vitamin D is present in certain foods and, as such, in cases of insufficient endogenous synthesis, its supply depends on its intake and, therefore, considered a vitamin. In foods, it can be in the form of cholecalciferol, if its origin is animal, or in the form of ergocalciferol (also called vitamin D2), if its origin is vegetable, being produced by fungi, yeasts, and some plants [1,2]. Both calciferols, in the liver, undergo a first hydroxylation at carbon 25, yielding, to simplify, 25-hydroxy-cholecalciferol (also named calcidiol or calcifediol) which is also biologically inactive. Calcidiol undergoes a second hydroxylation mainly, although not exclusively, in the kidneys. If this second hydroxylation occurs at carbon 1, 1,25-dihydroxy-cholecalciferol (also named calcitriol or 1,25-dihydroxyvitamin D (1,25(OH)$_2$D)) is produced, which is the true biologically active substance, and since it passes into circulation and determines multiple actions in different tissues it is, and should be called Hormone D [3]. As such hormone, it binds intracellular receptors called VDRs (Vitamin D Receptor) which are transcription factors that modify the expression of genes, the VDRE (Vitamin D Response Elements), which act in conjunction with other transcription factors [4]. The existence of VDR receptors has been described in practically all body tissues, which explains the pleiotropic nature of Hormone D [5–9]. Not all 25-hydroxy-cholecalciferol is transformed into 1,25-dihydroxy-cholecalciferol, most of it undergoes the second hydroxylation at carbon 24, originating 24,25-dihydroxy-cholecalciferol which is a substance with no known biological activity. In this text we will try to be precise in the use of the terminology, avoiding using the term vitamin D except when we refer to the nutritional source or we intend to include all the previous substances under a single denomination that, as we see, and by definition is in the strict sense wrong.

The most characteristic and classic action of Hormone D is to increase the supply of calcium, facilitating its intestinal absorption and kidney retention, and determining adequate bone mineralization and remodelation. Its deficiency causes, among other consequences, bone demineralization, which in adults is called osteomalacia, and in children rickets, which is accompanied by inadequate bone formation, insufficient growth, and susceptibility to infections [10,11]. However, as described below, calcitriol has more actions, and its lack determines various

consequences, some of which increase the susceptibility to various diseases and are associated with the aging process [12,13]. In this sense, its adequate supply (by skin synthesis or intake), the maintenance of optimal circulating levels, and the preservation of its correct functionality attenuate the loss of capacity and exhaustion of functional reserve that occurs with aging and, therefore, helps to prevent, at least in part, the consequences and complications of aging [14–16]. Consequently, it can be stated that calcitriol also has an anti-aging effect.

Interest in the role of vitamin D in aging has increased in recent years due to the high prevalence of vitamin D deficiency and the beneficial effects that its adequate supply and action have at a systemic level [13,14]. It is estimated that 40% of Western adults present a certain degree of hypovitaminosis D, which for the majority of them occurs without apparent clinical manifestations [17,18]. The present work focuses on the relationship between vitamin D/ calcidiol/ calcitriol and aging.

SOURCES AND METABOLISM

The different compounds related to vitamin D are secosteroids, derived from the structure of cyclopentane perhydrophenanthrene and, therefore, are fat-soluble. The common precursor is cholesterol, in fact, they derive from 7-dehydrocholesterol, which, by the concourse of sunlight, undergoes the opening of the B ring, rapidly forming previtamin D3 that more slowly transforms into vitamin D3 or cholecalciferol [19,20]. This process occurs in the skin level. Sun exposure of about 10 min a day on the arms and face is equivalent to the intake of 200 IU (about 5 mcg) and an exposure of a significant part of the skin for several hours a day is capable of generating up to 15000 IU of cholecalciferol [20]. However, there are differences depending on the individual phototype (more production in light-skinned people), latitude, season of the year, time of the day, use of sunscreens, age (lower in older people) [20,21]. Therefore, low circulating levels of calcidiol may occur despite adequate sun exposure and vice versa [22]. The amount of cholecalciferol produced or ingested can be expressed in International Units (IU) or in milligrams: 1 mg = 40,000 IU; 1 mcg = 40 IU; 1 IU = 25 ng.

Cholecalciferol is also present in the diet, it only comes from animal sources, but it is usually administered in the form of supplements. A compound with a structure very similar to cholecalciferol is ergocalciferol, also named vitamin D2. It is produced in yeasts and fungi also by the action of sunlight on ergosterol, present in their cell membranes and being the equivalent of cholesterol in animal cells [2]. Ergocholecalciferol is also a dietary source of vitamin D and is frequently the form of vitamin D present in supplements. As these substances are fat-soluble,

they require the presence of bile salts for their absorption, forming micelles in the digestive tract and absorbed with fat and incorporated into chylomicrons [23]. For this reason, and to facilitate vitamin D absorption, it is advisable that its intake be accompanied by a fatty meal. Absorption occurs mainly in the jejunum and to a lesser extent in the duodenum. Currently, the main sources of vitamin D are fish fat, eggs, butter and cod liver oil; some foods, such as milk, may also be enriched in this vitamin [10,24].

Given the fat-soluble nature of these substances, they circulate linked to a carrier protein, named DBP (Vitamin D Binding Protein) [25]. As that, enter the hepatocyte, where it undergoes a first hydroxylation by 25-hydroxylase becoming calcidiol. In the kidney, and to a lesser extent in other tissues, undergo a second hydroxylation at carbon 1 becoming calcitriol which is the active Hormone D [3]. The metabolization of these compounds is shown in Figure 1. Its excretion occurs via the bile after gluco- and sulfo-conjugation [26].
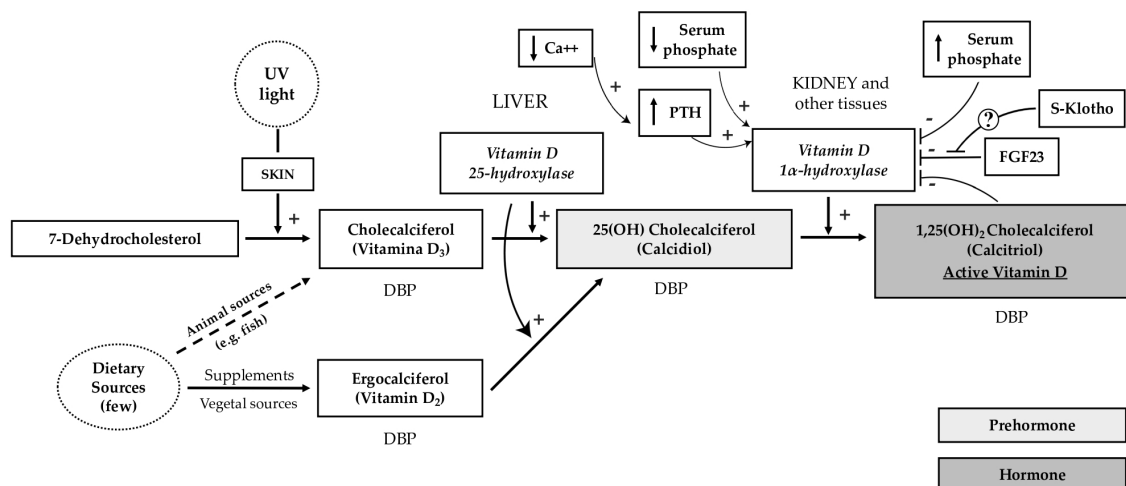
Figure 1. Synthesis and Regulation of Calcitriol

REGULATION OF SYNTHESIS

The synthesis of calcitriol, but not of calcidiol, is stringently regulated [8]. In this regulation intervenes the own calcitriol levels, by a sort of feed-back mechanism, as well as the plasma levels of calcium, phosphate, PTH (parathormone), and FGF23 (fibroblast growth factor 23) [8,27]. This regulation occurs by acting on two enzymes: 1-alpha-hydroxylase or CYP27B1 that determines the formation of 1,25-dihydroxy-cholecalciferol, the active hormone, and 24-hydroxylase or CYP24 that determines the formation of 24,25-dihydroxycholecalciferol, inactive [8,28]. Both enzymes are members of the mitochondrial P-450 system [29]. This process occurs mainly, but not exclusively, in the proximal convoluted tubules of the kidney, which express proteins that

facilitate the entry of DBP and, with that, calcidiol [8]. The decrease in calcitriol stimulates 1- and inhibits 24-hydroxylase. Increased calcitriol inhibits 1- and activates 24- hydroxylase. These enzymes are also directly regulated by phosphate: low phosphate stimulates 1- and inhibits 24-hydroxylase; high phosphate inhibits 1- and stimulates 24- hydroxylase. Calcium also regulates them through PTH. The decrease in calcium increases PTH, which stimulates 1- and inhibits 24-hydroxylase. Increased calcium inhibits PTH, which activates 24- and inhibits 1- hydroxylase. Increased calcitriol itself also inhibits PTH synthesis and secretion [28]. In other tissues, calcitriol synthesis is also regulated by VDRs [30].

Calcitriol stimulates, on the other hand, FGF23 which in turn inhibits 1- and stimulates 24-hydroxylase in a kind of second level feedback (-) mediated by this factor which, on its turn, controls both the levels of calcitriol and the availability of phosphate and calcium in a complex way. Thus, FGF23 decreases renal phosphate reabsorption, which counteracts the increase in intestinal phosphate absorption caused by calcitriol [27,31]. The alpha-Klotho protein is part of the FGF23 receptor and the soluble fraction of this receptor is the plasma S-Klotho protein, which circulation can be linked to FGF23 and modulate its biological effect [32–34].

MECHANISM OF ACTION

The mechanism of action of calcitriol is also complex. In general, it stimulates the expression of a series of genes involved in the transport and handling of calcium by cells, tissues and the whole body [35]. Since calcium is an important intracellular messenger, through this management calcitriol can exert many other actions and have multiple health consequences. To exert its actions, calcitriol binds to its intracellular receptor (VDR) which belongs to a subfamily of nuclear receptors that act as transcription factors in target cells after dimer formation with RXR (Retinoid X Receptor) [36]. VDRs have been identified in practically all the cells of the body, which explains the multiplicity of actions at the systemic level of calcitriol. Thus, more than 8000 binding areas, identified as VDR, have been characterized; areas that are also dynamic, and can vary during the process of cell differentiation, maturation and ageing as well as in cases of mutation, determining a multiplicity of effects on gene expression that, in addition, can vary from one circumstance to another [8,37,38].

Among the genes that are activated by calcitriol are those that express TPRV-type ion channels (transient receptor potential vanilloid type), specifically TRPV6 in the enterocyte [23,39]. Once TPRV is located in the luminal side of the plasma membrane, it will allow the gradient mediated passage of calcium from the lumen to the intestinal cell. In the proximal convoluted

tubule, and by a similar mechanism, but using TRPV5, calcitriol determines the reabsorption of the glomerular filtrated calcium [36,39]. Other calcitriol activated genes express calcium-binding proteins belonging to the troponin C superfamily [40]. A member of this family is Calbindin-D, which is found in the intestine, kidney or brain [23,41]. In the intestine, which is a well-established site of action of calcitriol, the expression of various types of calbindin-D allows calcium to be incorporated into the enterocyte [23]. This calcium leaves the enterocyte towards the interstitial space against gradient, a process mediated by a plasma membrane calcium-ATPase (PMCA) and a sodium/calcium exchanger (NCX) which expressions are also determined by calcitriol. This hormone also stimulates osteoblasts inducing collagen synthesis, alkaline phosphatase activity, and, hence, the accumulation and deposition of calcium with formation of bone hydroxyapatite, the bone mineral [42]. When the osteoblasts are stimulated by calcitriol, they, in turn, stimulate osteoclasts and, with it, the bone remodeling process [43]. But intestine, kidney, and bone are not the only action sites of calcitriol since its biological actions are pleiotropic [7–9,44].

ACTIONS. RELATIONSHIP WITH HEALTH AND WELL-BEING

Calcium momeostasis and bone physiology

Classically it has been considered that calcitriol acts in three target tissues: intestine, kidney, and bone [3,8,45]. In the intestine, it stimulates the absorption of calcium and phosphate, a process also influenced by diet and intestinal absorption capacity. In the kidney, determines calcium reabsorption at the tubular level. These two processes cause a greater supply and availability of calcium, but not inducing hypercalcemia due to the regulatory effect exerted by PTH. In bone, if the calcemia is normal, calcitriol determines bone mineralization and remodeling with activation of chondrocytes, osteoblasts, and, through them, osteoclast stimulation [3,8,45]. If calcemia is low, the joint action of PTH and calcitriol stimulates the differentiation of osteoclasts and consequently bone resorption [46,47]. One of the main risk factors for osteoporosis is precisely the deficiency of vitamin D and in its diagnosis, in addition to X-ray exams and bone densitometry, it is necessary to evaluate the circulating levels of calcidiol and, if possible, calcitriol [48].

Immune system and inflammation

Calcitriol has immunomodulatory effects, improving innate immunity and inhibiting an excessive adaptive immune response [49]. Dendritic cells, macrophages, and T and B lymphocytes express VDR but, in addition, most of these cells when stimulated in inflammatory processes

express 1-alpha-hydroxylase and, therefore, are locally capable of synthesizing calcitriol from calcidiol [49]. Calcitriol is also capable of inhibiting the expression of pro-inflammatory cytokines, acting directly on T cells to inhibit the secretion of IL-2, IFN-gamma, IL-6, IL-12, and TNF-alpha [49]. It also promotes the synthesis of antimicrobial peptides [49].

Cardiovascular system

Calcitriol is related to several factors that affect the cardiovascular function and health [50]. Calcitriol seems to be involved in the Wnt signaling pathway, responsible for cardiac cell differentiation, cell cycle control, blast formation in cardiovascular structures, and formation of myocardial fibers [51]. It also influences lipid metabolism, has a certain vasodilator action and antithrombotic activity, prevents calcium deposits, and the development of atherosclerosis [52]. On the other hand, as mentioned, there is a relationship between calcitriol levels, a reduction in pro-inflammatory elements (IL-1Beta) and an increase in anti-inflammatory interleukins (IL-10). This may positively influence the pathogenesis of stroke, mediated at the molecular level by the production of C-reactive protein (CRP), whose levels in the central nervous system have been shown to be reduced by calcitriol [53,54]. On the other hand, serum levels of calcitriol are also inversely correlated with the activity of the renin-angiotensin system and arterial hypertension [55].

Nervous system

Calcidiol and calcitriol, given their fat-soluble nature, cross the blood-brain barrier and it has been postulated that the microglia can form calcitriol in response to alterations in cell cycle regulation [56]. Calcitriol increases glutathione levels which can exert a neuroprotective and neuromodulatory effect [57]. The presence of 1-alpha-hydroxylase and VDR has also been observed in the midbrain and the substantia nigra, which suggests a potential relationship between calcitriol and dopaminergic neurons [58]. There is also VDR in the cerebellum, hippocampus, prefrontal cortex, and hypothalamus, so its deficit could be related to neurodegenerative diseases such as multiple sclerosis, Parkinson's, Alzheimer's, and even neuropsychiatric affective disorders such as depression [59]. In fact, calcitriol is capable of regulating the growth of oligodendrocyte-type neural stem cells and dopaminergic neurons, influencing the metabolism of dopamine, gamma-aminobutyric acid (GABA), and serotonin [60,61]. On the other hand, it has been reported that calcitriol is involved in the synthesis of neuronal growth factors involved in psychiatric pathologies with altered levels of serotonin such as depression, bipolar disorder,

schizophrenia, or personality disorders, some very sensitive to changes in exposure to sunlight [62,63]. Finally, calcitriol has actions that reduce cytotoxicity through the synthesis of neurosteroids in glial cells, attenuating the inflammatory response and reducing the activity of voltage-gated calcium channels involved in the generation of free radicals, thus preventing neurodegeneration [64].

Regulation of metabolism

Various studies suggest that calcidiol levels are inversely correlated with the risk of metabolic syndrome in healthy adults [65]. Thus, an almost 20% higher prevalence of hyperglycemia and abdominal obesity has been observed in patients with low serum levels of calcidiol, as well as higher levels of HDL-cholesterol in those who kept calcidiol within the optimal range (30-80 ng/ml) [65]. Vitamin D is fat-soluble so it can be stored in adipose tissue. Therefore, in obese people, its availability may decrease. In fact, an inverse relationship has been described between circulating levels of calcidiol and body mass index or body fat percentage [66]. On the other hand, adipocytes also contain VDR and it has been shown that calcitriol is capable, through calcium signaling, exert control on lipogenesis and lipolysis processes [67]. It has even been seen that calcitriol can modulate insulin action as well as its synthesis and secretion, which are determining factors in lipids and glucose metabolism [68]. In long-standing diabetic patients with neuropathic complications, the administration of cholecalciferol has resulted in improvements in metabolic control while improving quality of life, perception of illness or emotional stress, this being more evident in those patients who started from a deficiency state [68,69].

Digestive system and intestinal microbiome

Inflammatory bowel disease has been associated with low vitamin D levels and clinical improvements with its supplementation [70]. In fact, vitamin D is involved in the proper establishment of the intercellular junctions of the digestive epithelium [71]. On the other hand, vitamin D would also be involved in the regulation of the microbiota where VDRs have also been described [70–72]. The administration of probiotics increases the amount of both vitamin D and VDR in the intestinal cells, thereby determining an anti-inflammatory and protective effect against infections [73].

Respiratory apparatus

Vitamin D supplementation has been shown to decrease the risk of acute respiratory infections [74,75] and asthmatic exacerbations, which supports its immunomodulatory and anti-inflammatory character [76,77].

Skin

Vitamin D exerts regulatory effects on the proliferation of keratinocytes and anti-inflammatory properties are attributed at this level [78]. In psoriasis, there is a hyperproliferation of keratinocytes, for whose treatment the topical administration of analogues of this vitamin associated, or not, with corticosteroids has been proposed [79]. Vitamin D receptor has been proposed as a tumor suppressor in skin [80] and prevent against ultraviolet damage [81].

Oncology

Experimental studies have shown the association between low circulating levels of calcidiol and the risk of suffering from various types of cancer (colon, breast, prostate, ovary or skin) which may benefit from a vitamin D supplementation during treatment [82,83]. Several studies have shown that vitamin D has important regulatory roles of mechanisms controlling proliferation, differentiation, and growth. The administration of vitamin D analogues or the active metabolite of vitamin D activates apoptotic pathways, has antiproliferative effects, and inhibits angiogenesis [84]. This creates the potential for numerous therapeutic applications of vitamin D in diseases associated with auto-aggressive immune responses or in cancer [85].

CIRCULATING LEVELS

The circulating levels of calcidiol in healthy people are, or should be, around 30 ng/ml at least, and those of calcitriol around 0.03 ng/ml [86,87]. The circulating half-life of these compounds is very different, that of calcitriol is very short, about 4-6 h; that of calcidiol is very long, about 2-3 weeks; that of cholecalciferol and ergocolecalciferol is about 24 h [88]. Due to its higher concentration and high half-life, calcidiol is the more common analyte, but recently methods measuring calcitriol are available [86] although they are very scarce scientific papers reporting calcitriol plasma levels. There is no consensus regarding the optimal plasma level of calcidiol to prevent comorbidities. This is due to the different study designs, measurement methods, season of the year, age, sex, latitude or ethnicity [86]. Some definitions have been proposed [89–91] and adopted in most European

countries. These definitions consider plasma calcidiol values as: Optimal (> 50 ng / ml), Sufficient (20-50 ng / ml), Insufficient (<20 ng / ml) or Deficient: (<10 ng / ml).

The Spanish Society for Research in Mineral and Bone Metabolism indicates that calcidiol levels >30 ng/ml are adequate to ensure bone health, but higher plasma levels are required to ensure better health states [92]. A serum concentration of 20 ng/ml is the minimum desirable level in any age range. The levels currently proposed in Spain are:

- Optimal values: 30-80 ng/ml

- Insufficiency: 20-30 ng/ml

- Moderate deficiency: 10-20 ng/ml

- Severe deficiency (Rickets, Osteomalacia): <10 ng/ml

The main causes of vitamin D deficiency are [93]: (i) Alterations in intake: malnutrition, malnutrition, aging. (ii) Limited sun exposure: especially relevant in older institutionalized or dark-skinned patients. (iii) Malabsorption due to gastrointestinal disorders: Short Bowel Syndrome, IBD, Celiac disease, malabsorption after bariatric surgery, achlorhydria in elderly patients. (iv) Antiepileptic drugs: they alter the hepatic activity of 24-Hydroxylase transforming calcidiol into inactive metabolites. (v) Severe liver failure that impairs the function of 25-alpha-hydroxylase. (vi) Kidney failure or nephrotic syndrome that prevents the production of calcitriol. (vii) 1-alpha-hydroxylase failure: hypoparathyroidism, age-related kidney failure, enzyme deficiency (type I rickets). (viii) Resistance to vitamin D: rare autosomal recessive disorder with mutations in the vitamin D receptor (rickets type II).

DIAGNOSIS OF HYPOVITAMINOSIS D

Currently, universal screening is not indicated, its measurement being carried out only in the population at risk or in the aforementioned situations [94,95]. The diagnosis of the deficit is based on the confirmation of low serum levels of calcidiol and the performance of complementary tests that confirm a low bone mineral density, being double emission X-ray absorptiometry (DXA) the reference method. Clinical data suggestive of vitamin D deficiency include diffuse and deep bone pain, that is accentuated with physical activity and more intense in lower limbs and lumbar and pelvic areas; asthenia and arthromyalgia; tetany; pathological fractures at vertebra, ribs, intertrochanteric or metatarsal [94,95]. In laboratory data can be found: low or close to the lower limit of normality calcemia and phosphataemia, elevated levels of alkaline phosphatase and PTH, and hypocalciuria, among others. In radiology exams: alterations in the trabecular pattern, acetabular

protrusion, densitometry T-score <-2.5, diffuse or local scintigraphic pattern in pseudofractures [96].

SUPPLEMENTATION

Given that the plasma range of calcidiol considered more adequate for the general population is 30-80 ng/ml (75-200 nmol/L), and levels below 100 ng/ml (250 nmol/L) are generally considered as safe, the administration of around 4,000 IU (100 mcg) seems adequate [97,98] although also challenged [99] but more important than the unitary dose is the cumulative dose [98] because, as a fat-soluble substance, it enters the cells and accumulates in the adipose tissue from where it can be later released. The Spanish Osteoporosis Society establishes that in patients at risk, a repeated supplementation of cholecalciferol should be kept between 1,000 IU and 5,000 IU per day, which would allow achieving circulating levels of about 120 ng/ml [92]. Annual administrations of more than 500,000 IU are not recommended as bone loses elasticity, increasing the risk of falls and pathological fractures [100]. Regardless of the form of supplementation, periodic analytic controls are always recommended [97–99].

For supplementation with cholecalciferol, the following guidelines are recommended:

- In cases of severe deficiency (<10ng / ml), 25,000 IU twice a week for 6 weeks, followed by 25,000 IU per week for 4 weeks and a maintenance dose of 25,000 IU every 2-4 weeks according to the risk profile.

- In cases of moderate deficiency (10-20ng / ml), 25,000 IU per week for 8 weeks and every two weeks for the following 8 weeks. A dose of 25,000 IU can be maintained every 2-4 weeks according to risk level and cumulative dose.

- In case of insufficiency (20-30ng / ml), 25,000 IU every 2 weeks for 4 weeks and continue with 25,000 IU per month.

If calcidiol (calcifediol) is used as a supplement, similar serum values can be achieved with half of the above proposed doses [98]. The administration of this compound is mandatory in patients with liver disease where hepatic hydroxylation is compromised. With this form of supplementation, it is necessary to have a stricter control of circulating levels since it can displace calcitriol from DBP, increasing the active form in circulation and thus its possible toxicity [98].

Close monitoring is required in patients receiving high doses of vitamin D (10,000 to 50,000 IU) [97]. Overdose produces hypercalcemia, anorexia, thirst, weakness, mental disconnection, polydipsia, polyuria, nephrocalcinosis or even fatal arrhythmias [101]. To date, vitamin D toxicity poisoning has been documented with the administration of 60,000 IU per

week. Treatment includes immediate drug withdrawal, as well as life support measures: rehydration, diuretics, calcitonin, corticosteroids, renal function monitoring, and ECG [101]. Vitamin D accumulates in fatty tissue so that the intoxicated patient must be monitored for some time levels as the hormone is progressively released into the circulatory system [101].

VITAMIN D, AGING, ANTI-AGING AND KLOTHO PROTEIN

Taking into consideration the biological actions described above, calcitriol can contribute to a more healthy and active ageing [12,14]. A consequence of age is precisely the loss of bone and muscle mass, which together with a lower coordination capacity and longer perception/reaction times determine greater fragility and increased risk of falls that occasionally may lead to serious fractures that require surgical interventions. Maintaining optimal calcidiol levels (30-80ng / ml) is associated with a decrease in the incidence of fractures in people over 65 years of age [102–105] and also improves the postoperative period in those patients who have suffered complicated fractures requiring surgery [106].

Various studies have also revealed the relationship between the dose of vitamin D and the prevention of functional decline in elderly patients, one of its main exponents is sarcopenia, which implies a loss of strength and muscle mass [107]. In a double-blind, randomized, controlled clinical trial conducted in people over 65 years old with sarcopenia, where part of the intervention consisted of administering 800 IU/day of vitamin D, it was shown that calcidiol concentrations in blood above 20 ng/ml, together with a protein intake adjusted to the needs and the practice of programmed physical activity are required to effectively fight sarcopenia [108]. Accumulating Evidence stress the importance of vitamin D Supplementation to Prevent COVID-19 Infections and Deaths [109].

During aging, resistance to the action of calcitriol occurs due to the decrease in the number of VDR, which leads, among other effects, to a lower intestinal absorption of calcium [105]. Added to this is the loss of kidney function, which implies less activity of 1-alpha-hydroxylase and less calcitriol synthesis [105]. This deficit influences the aging process itself at multiple levels [110], and it has been shown that supplementation with cholecalciferol is associated with a reduction in all-cause mortality [111].

One of the mechanisms by which calcitriol may influence aging is through the expression of the Klotho gene [112]. The joint action of Klotho and calcitriol maintain an adequate regulation of the cell cycle through P21, and exert antioxidant effects via FOXO and FGF23 homeostasis, all of which implies a protective effect against malignant and/or degenerative processes [31,32]. Thus,

recent studies have related alpha-Klotho with ATP mediated Na/K handling, is also involved in calcium homeostasis through influencing the synthesis of PTH. Similarly, Klotho could regulate the synthesis of FGF23 which in turn controls the availability of calcium and phosphate [31–33]. On the other hand, calcitriol, by binding VDR at the renal level, modifies the expression of Klotho and Nrf2 genes [31–33]. The calcitriol / Klotho / Nrf2 complex is capable of maintaining correct calcium homeostasis and control of oxidative damage, playing a protective role against degenerative alterations linked to aging in multiple tissues [33]. It has also been shown that Klotho inhibits the Insulin / IGF-I signaling pathway, favoring autophagy and consequently exerting a protective role against metabolic disorders particularly those associated with aging [33]. This is believed to be mediated by inhibition of the FOXO protein. Klotho has also been linked to the inhibition of the Wnt pathway which is related to cell cycle prolongation, accumulation of mutations, and fibrosis in kidney cells and possibly other cells [113]. Lastly, the soluble and circulating form S-Klotho can have direct anti-ageing effects, protecting against skin atrophy, osteopenia, hyperphosphatemia, endothelial disorders, neurodegeneration, and also activate various antioxidant pathways possibly by blocking FGF23 [114] and calcitriol may play a pivotal role [115]. The literature in this regard is scarce and more studies are needed to address the interrelation between calcitriol, S-Klotho protein, and interventions and circumstances with anti-aging effects.

CONCLUSION

Vitamin D deficiency and low circulating levels of calcitriol are highly prevalent in the general population and this may have important social and health consequences. Various studies have shown that optimal levels of calcidiol (30-80 ng/ml) have a protective effect not only at the bone level but also at the muscular, neurological, cardiovascular, gastrointestinal, metabolic, immune, oncological, and even anti-aging levels. Adequate sun exposure, the consumption of foods rich in vitamin D, and eventually the supplementation with cholecalciferol, or even calcidiol, will have a beneficial effect on health and well-being and will contribute to more active and healthy aging. Studies are needed that specifically analyze the levels of the active hormone, calcitriol, and its relationship with those factors and circumstances that are associated with healthier aging.

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AIMS

The overall aims of this International Doctoral Thesis were: (i) to study the association of $1,25(OH)_2D$ with body composition, physical activity levels, physical fitness, cardiometabolic health, and the S-Klotho protein, and (ii) to study the effect of different exercise training modalities on $1,25(OH)_2D$ in middle-aged sedentary adults. These overall aims are addressed in five different studies which are grouped in two sections.

SECTION 1: 1,25-dihydroxyvitamin D and ageing markers: body composition, physical activity levels, physical fitness, cardiometabolic health, and S-Klotho protein

- **General objective 1:** To examine the association of $1,25(OH)_2D$ with body composition, physical activity levels, physical fitness, cardiometabolic health, and S-Klotho protein in middle-aged sedentary adults.

  - *Specific objective 1.1:* To study the relationship of $1,25(OH)_2D$ with body composition including lean and fat body mass as well as bone mineral density in middle-aged sedentary adults **(Study 1)**.

  - *Specific objective 1.2:* To investigate the relationship of sedentary time, physical activity levels, and physical fitness (i.e., maximal oxygen uptake and muscular strength) with $1,25(OH)_2D$ in middle-aged sedentary adults **(Study 2)**.

  - *Specific objective 1.3:* To investigate the relationship of $1,25(OH)_2D$ with cardiometabolic risk factors in middle-aged sedentary adults. **(Study 3)**.

  - *Specific objective 1.4:* To investigate the relationship between $1,25(OH)_2D$ and S-Klotho in middle-aged sedentary adults, as well as to study the mediation role of body composition in the association between $1,25(OH)_2D$ and S-Klotho **(Study 4)**.

SECTION 2: Role of exercise on 1,25-dihydroxyvitamin D

- **General objective 2:** To investigate the effects of different exercise training modalities on $1,25(OH)_2D$ in middle-aged sedentary adults.

  - *Specific objective 2.1:* To study the effects of 12 weeks different training modalities ([a] a concurrent training based on physical activity recommendation from the World Health Organization group, [b] a high-intensity interval training group, and [c] a high-intensity interval training group adding whole-body electromyostimulation group) on $1,25(OH)_2D$ in

middle-aged sedentary adults as well as to examine whether these hypothetical changes in 1,25(OH)$_2$D are associated with changes in body composition and physical fitness **(Study 5)**.

MATERIAL AND METHODS

All the content regarding the material and methods of the present International Doctoral Thesis has been based on the previously published methodological article on the FIT-AGEING study [1], in which the author of the present Doctoral Thesis appears as the first co-author. This methodological article was included as a chapter in a previous International Doctoral Thesis [2].

DESING

The present doctoral thesis were conducted under the framework of the FIT-AGEING study, an exercise-based randomized controlled trial (ClinicalTrials.gov ID: NCT03334357) [1]. The study protocols, experimental design and informed consent procedure were approved by the Ethics Committee on Human Research of the Regional Government of Andalucía [0838-N-2017]. Prior to any data collection, all participants provided written informed consent after having received and understood the details of the study assessments and intervention. Following baseline testing, participants were randomly allocated into four different training groups: (1) a control group (no exercise), (2) a concurrent training based on physical activity recommendation from the World Health Organization (PAR) group, (3) a high intensity interval training (HIIT) group, and (4) a high intensity interval training adding whole-body electromyostimulation (HIIT+EMS) group. All groups were followed over a period of 12 months. All of the baseline and post-intervention measurements were performed in the same setting [*Centro de Investigación Deporte y Salud* (*CIDS*, Granada, Spain) and at the "Campus de la Salud" Hospital (Granada, Spain)]. The study followed the last revised Ethical Principles for Medical Research Involving Human Subjects comprised in the Declaration of Helsinki [3] and was conducted into two waves (September–December 2016 and September-December 2017) of forty-five participants maximum due to reasons of feasibility and practicality, as well as to avoid any potential seasonal bias. FIT-AGEING study had highly standardized protocols for selection criteria, recruitment, exercise training programs, outcomes measurements, and data collection.

Participants and eligibility

Eighty-nine middle-aged sedentary adults (52.7% women) were voluntarily enrolled in the FIT-AGEING study. The participants should be adults from the province of Granada (Spain) with 45-65 years old and should have a BMI between 18.5 to 35 kg/m^2. Including people with different weight status and body composition allows to study 1,25(OH)$_2$D differences across BMI categories (i.e., normal-weight, overweight and obese) and/or on amount of fat body mass. A full list of inclusion and exclusion criteria for participants is defined in Table 1.

Table 1. Eligibility criteria

Inclusion criteria	Exclusion criteria
- Age: 45-65 years old	- History of cardiovascular disease
- BMI: 18.5-35 kg/m²	- Diabetes mellitus
- To be sedentary (less than 20 minutes of moderate-intensity physical activity on 3 days/week over the last three months)	- Pregnancy or planning to get pregnant during the study period.
- Not participating in a weight-loss program	- Beta blockers or benzodiazepines use
- To have a stable weight over the last three months (body weight changes <3kg)	- Taking any medication
- Participants must be capable and willing to provide consent, understand exclusion criteria and accept the randomized group assignment	- Unwillingness to either complete the study requirements or to be randomized into the control or training group
	- To have a major illness (acute or chronic) including any that would limit the ability to perform the necessary exercises

BMI: Body mass index.

Prior to the enrollment, all potential individuals completed a health history revision and were medically examined to identify any pathological condition and current medication that could affect the ability to complete the required assessment protocols and intervention exercise programs. If any medical problems appeared during the intervention, participants were referred for medical evaluation and, if necessary, dropped from the study.

Recruitment

The recruitment of participants was performed using different strategies including the use of social networks, local media, word of mouth, and posters at different points of Granada. Furthermore, information meetings were organized at the School of Medicine of the University of Granada. People interested contacted the research staff through phone and/or e-mail to provided general information about the study. They visited the research center to receive a thorough explanation about the study aims, inclusion and exclusion criteria, assessment to be performed, study requirements of the participants, and types and characteristics of the intervention programs. After clarification by the research staff of any participant's questions or doubts, the potential participants meeting the inclusion criteria were invited to a second orientation session. In this session, the participants received detailed written information about the study methodology, and were asked to sign the informed consent. Finally, the participants were cited and informed of all necessary preconditions for their first assessment day.

Figure 1 shows the participants flow diagram from the recruitment to the randomization stages of this study.
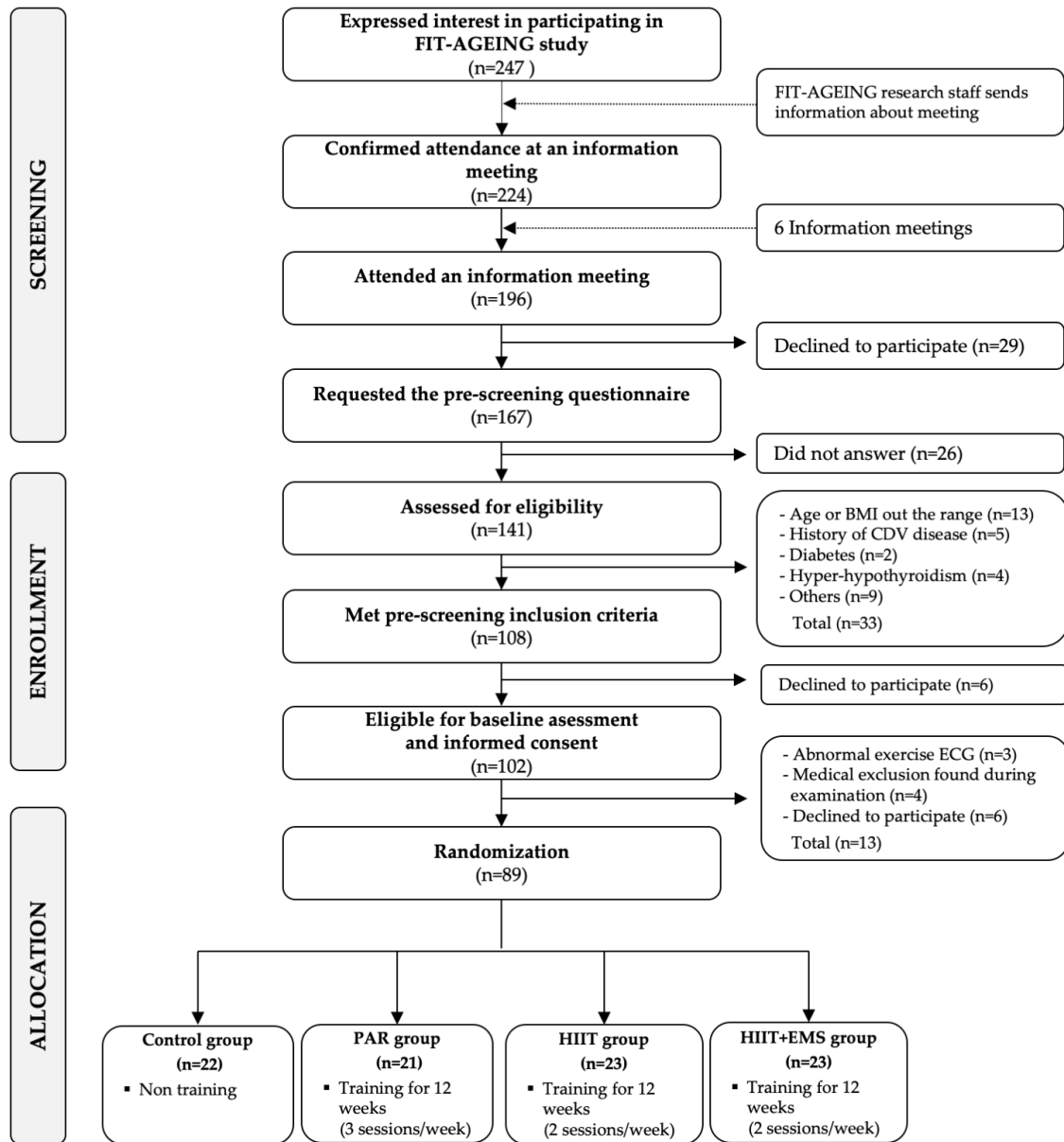
Figure 1. Flow diagram of the FIT-AGEING study participants. BMI: Body mass index; CDV: Cardiovascular; ECG: Electrocardiogram; PAR: Concurrent training based on physical activity recommendation from the World Health Organization; HIIT: High intensity interval training; HIIT+EMS: High intensity interval training adding whole-body electromyostimulation.

Randomization and blinding

Once included in the study, the participants received an internal number to be de-identified. After completing the baseline measurements, the eligible participants were randomly allocated to either a control group or an exercise training group. Sequence of allocation was based on computer-generated using simple randomization [4]. Due to the nature of interventions, the research staff conducting exercise training sessions was not blinded. Each participant was specifically informed of their assigned group. In this sense, to ensure masking of the assessment staff, participants were frequently reminded to omit their assigned group and not talk about their interventions during follow-up measurements sessions. For practical and feasibility reasons, the study was conducted in two waves of a maximum of 45 participants.

Sample size

The determination of the sample size and power calculation of FIT-AGEING study were computed based on its primary outcome (S-Klotho plasma levels), based on a pilot sample [5]. The research staff considered S-Klotho plasma levels differences between pre- and post-treatment in order to assess the sample size requirements for the one-way ANOVA [6]. To meet these criteria, a minimum of 14 participants per group was predicted to provide a statistical power of 85% considering a type I error of 0.05. Assuming a maximum loss at follow-up of 25%, the research staff decided to recruit a minimum of 20 participants (approximately 50% women) for each study group: control, PAR, HIIT, and HIIT+EMS. IBM-SPSS Sample power software (version 3.0.1) was used for calculations.

Participant retention and adherence

The participants were allowed to withdraw at any time throughout the intervention study; however, in order to reduce drop-out cases and to maintain adherence to the training program, every effort was made to provide a positive training environment: (i) all sessions were accompanied by music that participants could choose, and were held on an airy, well equipped, and well-lighted gym; (ii) qualified and certificated trainers were carefully supervising every session; (iii) the training sessions were conducted in small groups (≤6 persons) to ensure that participants were performing the exercises correctly at an adequate intensity; (iv) different training schedules were offered in all groups to fit each participant's needs; (v) if assistance fell below target (i.e., 90%), the exercise trainer worked with the participant to identify barriers and

increase adherence; (vi) the intervention program was carried out from September to December to avoid vacations periods that might interfere with participant's availability; (vii) we used phone calls to inquire for any adverse events if a participant missed a session. These and other strategies such as positive reinforcement, regular follow-up, and supported the participants were used by training specialist and the other study staff to enhance study adherence.

EXERCISE TRAINING PROGRAMS

In order to compare various exercise intensity levels (moderate vs. high intensity) to test if higher intensity levels provide greater positive effects on the variables analyzed despite the application of a lower training volume, different exercise training modalities were applied in the FIT-AGEING study for 12 weeks [1]. This length in the intervention program was proposed based on the results of a previous randomized clinical trial [7], and considering that the substantial physiological adaptations occur within the first 12-24 weeks of exercise training [8]. A full description of each exercise training program can be found in the previously published methodological article of FIT-AGEING study [1]. Briefly, the exercise training programs belonging to study 5 of the present International Doctoral Thesis were designed as follows:

Concurrent training based on physical activity recommendation from the World Health Organization (PAR)

Concurrent training is defined as the combination of aerobic and resistance training. Based on both types of training, the World Health Organization (WHO) proposed the Global Recommendations on Physical Activity for Health, which focus is primary prevention of noncommunicable diseases through physical activity at population level [9]. These recommendations were established independently for different age ranges. In this sense, our training program was based on a previous randomized controlled trial, which had the aim to meet these physical activity recommendations in adults [7].

<u>Volume, intensity, frequency and type of exercise</u>

The participants allocated to the PAR group completed 3 concurrent training sessions per week for 12 consecutive weeks with at least 48 hours of recovery between sessions. This training program was based on the minimum physical activity recommended by the WHO [9]. As such, the training volume was 150 min/week at an intensity of 60-65% of the heart rate reserve for the aerobic training, and ~60 min/week at an intensity of 40-50% of one-repetition maximum was

established for the resistant training. The aerobic training section was performed using a treadmill, cycle-ergometer, and elliptical ergometer exercises. For the resistance training section, weight bearing and guided pneumatic machines were used, such as bench press, squat, lateral pull down or dead lift, among others. In order to reduce the risk of injuries, as well as to promote exercise adherence, compensatory exercises (flexibility, stabilizer muscles, and core stability) were included.

Training sessions

The PAR intervention program was organized in two types of sessions, combined training session (aerobic and resistance training) and non-combined training session (only aerobic training).

All combined and non-combined training sessions began with a dynamic standardized warm-up, including several muscle activation exercises. After warm-up on the combined sessions, an aerobic exercise was carried out on 10-minute sets, alternating with resistance exercises, which varied depending on the session (see Table 2). The participants had the possibility to change the ergometer in different 10-minute aerobic sets (cycle-ergometer, treadmill, or elliptical). In non-combined sessions the participants had to complete a total of 60 minutes of aerobic exercise. In addition, these sessions included compensatory exercises.

In all cases, the participants ended the session with a cooling-down protocol (active global stretching), completing five anterior and posterior chain exercises.

Training load variation

Due to the sedentary condition of the participants, a gradual progression of the training program was proposed in order to control the exercise dose and achieve the volume and intensity required (see Table 2). The training program progressed as follows:

- *Aerobic training:* The training program started with an aerobic dose of 75 min/week at 60% of the heart rate reserve (HRres), which was progressively increasing 30 min/week until achieving a total of 150 min/week on the 4th week.

- *Resistance training:* The training program started with a two weeks familiarization phase, whose main objective was that the participants to learn the movement patterns necessary to perform the resistance exercises of this specific training program. Furthermore, compensatory exercises were included to improve core competency and joints stabilization, in order to avoid injuries.

An important aspect related to variation in training load is the increase of the participants' fitness level that occurs as the intervention program progresses, which leads to a necessary increase of aerobic and resistance load. In this sense, the previously established aerobic training intensity was progressively increasing when the participant's physical fitness was increased, in order to maintain a specific percentage of the HRres. Moreover, in order to adjust the resistance training load, the 1RM measurement of all exercises was performed in the first week of each phase of the intervention program.

On the other hand, two types of sessions were included in each training phase in order to provoke different physiological adaptations in terms of muscle hypertrophy (muscle damage, metabolic stress, etc.), and consequently a greater stimulus in the dependent variables analyzed in the study.

Training periodization

The training periodization of PAR program can be seen in Table 2. It was divided into three phases:

(i) *Familiarization phase:* Its duration was of 2 weeks. The principal aim of this phase was to learn the main movement patterns (hinge, bridge, squat, and horizontal and vertical pulls and push), as well as to improve many physical fitness components (i.e., cardiorespiratory fitness, core stability, joint stabilizing muscles, balance, and flexibility). This phase was essential to prepare the participants for the 1RM evaluation.

(ii) *Phase I:* Its duration was of 5 weeks. In this phase, the participants performed two combined sessions (aerobic and resistance training), and only one aerobic training session each week as follow:

- The aerobic training: The volume was 150 min/week (except in RM weeks, with a duration of 120 min/week), and the intensity selected was 60% of the HRres in all cases.

- The resistance training: Exercises involving the major muscle groups and principal movement patterns (i.e., bench press, squat, lateral pull down, dead lift…) and compensatory exercises were included.

Combined sessions were structured following the Type I session, which alternated 4 resistance exercises that involved the major muscle groups, 2 core stability exercise, and 2 compensatory exercises with 10-minute sets of aerobic training.

(iii)   *Phase II:* Its duration was of 5 weeks. In this phase, the combined sessions were organized differently in order to provide a different resistance training stimulus [9–11]. These sessions were divided as follow:

- Type I session: It was focused on mechanical tension and muscle damage.
- Type II session: It was focused on metabolic stress.

The exercises included in both sessions were similar to those reported in phase I. Furthermore, several exercises that involved the small muscle groups (i.e., lateral raises or French press) were also included.

MATERIAL AND METHODS

Table 2. PAR training periodization for 12 weeks

	AEROBIC TRAINING			RESISTANCE TRAINING	
Weeks	**Volume (min)**	**Intensity (% HRres)**		**Intensity (% RM)**	**Type of exercise**
FAMILIARIZATION PHASE					
Week 1	75	60		Weight-bearing and elastic band	Movement pattern and
Week 2	105	60			global movements
PHASE I					
Week 3	120	60		1 RM ASSESTMENT	
Week 4	150	60		50	Exercises involving major
Week 5	150	60		50	muscle groups
Week 6	150	60		50	
Week 7	150	60		50	
PHASE II					
Week 8	120	60		1 RM ASSESTMENT	
Week 9	150	60		50	Exercises involving major
Week 10	150	60		50	muscle groups
Week 11	150	60		50	
Week 12	150	60		50	

PAR: Concurrent training based on physical activity recommendation from the World Health Organization; Min: Minutes; HRres:
maximum.

Table 3. Combined training session in the PAR training program

SESSION TYPE I				SESSION TYPE II			
Exercise	Sets	Volume	Intensity	Exercise	Sets	Volume	Intensity
WARM-UP							
Aerobic Warm-up	1	5 min	60% HRres	Aerobic Warm-up	1	5 min	60% HRres
Dynamic Warm-up	1	5 min		Dynamic Warm-up	1	5 min	
MAIN PART							
Aerobic I	1	10 min	60% HRres	Aerobic I	1	10 min	60% HRres
Resistance Exercise I	1	10 reps	40-50% RM	Aerobic II	1	10 min	60% HRres
Resistance Exercise II	1	10 reps	40-50% RM	Resistance Exercise I	1	10 reps	40-50% RM
Resistance Exercise III	1	10 reps	40-50% RM	Resistance Exercise V	1	10 reps	40-50% RM
Resistance Exercise IV	1	10 reps	40-50% RM	Resistance Exercise II	1	10 reps	40-50% RM
Aerobic II	1	10 min	60% HRres	Resistance Exercise VI	1	10 reps	40-50% RM
Resistance Exercise I	1	10 reps	40-50% RM	Resistance Exercise III	1	10 reps	40-50% RM
Resistance Exercise II	1	10 reps	40-50% RM	Resistance Exercise VII	1	10 reps	40-50% RM
Resistance Exercise III	1	10 reps	40-50% RM	Resistance Exercise IV	1	10 reps	40-50% RM
Resistance Exercise IV	1	10 reps	40-50% RM	Resistance Exercise VIII	1	10 reps	40-50% RM
Aerobic III	1	10 min	60% HRres	Aerobic III	1	10 min	60% HRres
Resistance Exercise I	1	10 reps	40-50% RM	Aerobic IV	1	10 min	60% HRres
Resistance Exercise II	1	10 reps	40-50% RM	**COOL-DOWN**	1	5 min	
Resistance Exercise III	1	10 reps	40-50% RM				
Resistance Exercise IV	1	10 reps	40-50% RM				
Aerobic IV	1	10 min	60% HRres				
Resistance Exercise I	1	10 reps	40-50% RM				
Resistance Exercise II	1	10 reps	40-50% RM				
Resistance Exercise III	1	10 reps	40-50% RM				
Resistance Exercise IV	1	10 reps	40-50% RM				
COOL-DOWN	1	5 min					

PAR: Concurrent training based on physical activity recommendation from the World Health Organization; HRres: Heart rate reserve; RM: Repetition maximum; Min: Minutes; Reps: Repetitions.

High intensity interval training (HIIT)

HIIT training, defined as intervals of work involving brief vigorous-intensity physical exercise interspersed by intervals of recovery (at a lower intensity or passive rest), has been suggested as an effective strategy to get the same or even higher improvements in physiological and health-related markers in healthy and diseased populations in comparison to moderate-intensity exercise [12–14]. These findings are important from a public health perspective since HIIT overcomes one of the most common personal barriers to physical exercise training such as "lack of time" [15].

Volume, intensity, frequency and type of exercise

The participants allocated to the HIIT group completed 2 sessions per week for 12 weeks with at least 72 hours of recovery between sessions considering the age of the participants (45-65 years old) and their training level (sedentary). This training program involved two different and alternative high intensity interval training protocols [16,17], which included a high intensity interval

training with long intervals (type A session), and a high intensity interval training with short intervals (type B sessions). Type A session was composed by a training volume of 40-65 min/week at >95% of the maximum oxygen uptake (VO$_2$max). Treadmill exercise with a personalized slope was chosen for this protocol. Type B session was composed by a training volume of 40-65 min/week at level 6-9 on a perceived maximum effort scale [ranged from 0 to 10] [18]. Eight weight-bearing programmed exercises in circuit form (i.e., squat, dead lift, high knees up, high heels up, push up, horizontal row, lateral plank, and frontal plank) was included in this protocol.

Training session

The HIIT intervention program was organized in two types of sessions:

- *Type A session:* It started with a dynamic standardized warm-up, including several muscle activation exercises. Subsequently, the participants performed 5 minutes of aerobic exercise on the treadmill at 60% of VO$_2$max. After the warm-up, the participants performed several treadmill sets following the established parameters and periodization described below (see Table 4).

- *Type B session:* It started with a dynamic standardized warm-up. After the warm-up, the participants performed a circuit training composed of eight weight-bearing exercises, twice per set with an active rest (walking at 60% of VO$_2$max) following the established parameters and periodization described below.

Training load variation

Due to the sedentary condition of the participants, a gradual progression of the training program was proposed in order to control the exercise dose and achieve the volume and intensity required. Thus, the different types of sessions progressed as follows:

- *Type A session:* The participant started with a dose of <40 min/week at 80-90% of VO$_2$max (familiarization phase). It was progressive increased to 50 min/week at >95% of VO$_2$max (phase I), and to 65 min/week at >95% of VO$_2$max (phase II).

- *Type B session:* The participant started with a dose of <40 min/week at 80-90% of VO$_2$max (familiarization phase). It was progressive increased to 50 min/week at 120% of VO$_2$max (phase I), and to 65 min/week at 120% of VO$_2$max (phase II).

Training periodization

The training periodization of HIIT program can be seen in Table 4. It was divided into three phases of four weeks each, and two type of sessions each week (type A and type B):

(i) *Familiarization phase:* The intensity selected for the first two weeks was 80% of VO$_2$max, completing 6-7 sets of 4 minutes (2 minutes work/2 minutes rest) with a maximal duration of 14 minutes/session in session type A. In session type B, the participants completed 2 sets (8-10.5 min) of 16 exercises (15-20 seconds work/ 15-20 seconds rest) with an active rest of 5 min at 60% VO$_2$max between each set, and a maximal duration of 21 min/session. In the third and fourth week both intensity and volume were increased. Thus, the intensity selected was 90% of VO$_2$max, completing 8-9 sets of 4 minutes (2 minutes work/ 2 minutes rets) with a maximal duration of 18 minutes/session in session type A. The session type B maintained the same volume of work and rest in the previous two weeks.

(ii) *Phase I:* The intensity selected was >95% VO$_2$max, completing 8-10 sets of 4 minutes (2 minutes work/2 minutes rest) with a maximal duration of 20 minutes/session in session type A. In session type B, the intensity was 120% VO$_2$max, completing 2 sets (8-16 min) of 16 exercises (15-30 seconds work/ 15-30 seconds rest) with an active rest of 5 min at 60% VO$_2$max between each set, and a maximal duration of 32 min/session.

(iii) *Phase II:* In this phase, the intensity was the same compared to phase I in both types of sessions (>95% VO$_2$max, and 120% VO$_2$max respectively). However, training volume was higher than phase I but not exceeding 65 minutes/week. In session type A, the participants completed 6-8 sets of 5 minutes (3 minutes work/2 minutes rest) with a maximal duration of 24 minutes/session. In session type B, the participants completed 3 sets (8-13.5 min) of 16 exercises (15-30 seconds work/ 15-30 seconds rest) with an active rest of 5 min at 60% VO$_2$max between each set, and a maximal duration of 40.5 min/session.

MATERIAL AND METHODS

Table 4. HIIT training program periodization for 12 weeks

	FAMILIARIZATION PHASE					
Week	**1**		**2**		**3**	
Session (Type)	1 (A)	2 (B)	3 (A)	4 (B)	5 (A)	6 (B)
Exercises	1 (Tr)	8x2 =16 (W-B)	1 (Tr)	8x2 =16 (W-B)	1 (Tr)	8x2 =16 (W-
Volume	12 min	16 min	14 min	21 min	16 min	16 min
Intensity	80% VO_2max	80% VO_2max	80% VO_2max	80% VO_2max	90% VO_2max	90% VO_2m
Sets	6	2	7	2	8	2
Set duration	4 min	8 min	4 min	10.5 min	4 min	8 min
Work exercise	2 min	15 Sec	2 min	20 Sec	2 min	15 Sec
Rest exercise	2 min (passive)	15 Sec	2 min (passive)	20 Sec	2 min (passive)	15 Sec
Rest between sets	—	5 min (60% VO_2max)	—	5 min (60% VO_2max)	—	5 min (60% VO
	PHASE I					
Week	**5**		**6**		**7**	
Session (Type)	9 (A)	10 (B)	11 (A)	12 (B)	13 (A)	14 (B)
Exercises	1 (Tr)	8x2 =16 (W-B)	1 (Tr)	8x2 =16 (W-B)	1 (Tr)	8x2 =16 (W-
Volume	16 min	16 min	18 min	21 min	20 min	27 min
Intensity	>95% VO_2max	120% VO_2max	>95% VO_2max	120% VO_2max	>95% VO_2max	120% VO_2m
Sets	8	2	9	2	10	2
Set duration	4 min	8 min	4 min	10.5 min	4 min	13.5 min
Work exercise	2 min	15 Sec	2 min	20 Sec	2 min	25 Sec
Rest exercise	2 min (passive)	15 Sec	2 min (passive)	20 Sec	2 min (passive)	25 Sec
Rest between sets	—	5 min (60% VO_2max)	—	5 min (60% VO_2max)	—	5 min (60% VO
	PHASE II					
Week	**9**		**10**		**11**	
Session (Type)	17 (A)	18 (B)	19 (A)	20 (B)	21 (A)	22 (B)
Exercises	1 (Tr)	8x2 =16 (W-B)	1 (Tr)	8x2 =16 (W-B)	1 (Tr)	8x2 =16 (W-
Volume	18 min	24 min	21 min	31.5 min	24 min	40.5 min
Intensity	>95% VO_2max	120% VO_2max	>95% VO_2max	120% VO_2max	>95% VO_2max	120% VO_2m
Sets	6	3	7	3	8	3
Set duration	5 min	8 min	5 min	10.5 min	5 min	13.5 min
Work exercise	3 min	15 Sec	3 min	20 Sec	3 min	25 Sec
Rest exercise	2 min (passive)	15 Sec	2 min (passive)	20 Sec	2 min (passive)	25 Sec
Rest between sets	—	5 min (60% VO_2max)	—	5 min (60% VO_2max)	—	5 min (60% VO

Type A: High intensity interval training on the treadmill with individual slope; Type B: High intensity interval training (weight-be
bearing exercises; VO_2max: Maximal oxygen uptake; Min: Minutes; Sec: Seconds.

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High intensity interval training adding whole-body electromyostimulation (HIIT+EMS)

Whole-body electromyostimulation (WB-EMS) has recently emerged as an innovative training modality, and enables the simultaneous exogenous stimulation of up to 14-18 regions or 8-12 different muscle groups (i.e., upper legs, upper arms, gluteals, abdomen, chest, lower back, upper back, and shoulder) with up to 2800 cm^2 electrode area [19]. It allows the configuration of different intensities in each region. For the use of this training technology is essential to follow the scientific recommendations related to WB-EMS to avoid irresponsible use that can lead to health problems [20,21].

In this sense, given that the participants have never done WB-EMS, the research staff established a gradual and progressive WB-EMS training periodization in order to avoid possible dangerous health consequences [22,23].

Volume, intensity, frequency, type of exercise, training sessions and periodization

The participants allocated in the HIIT+EMS group completed a training program following the same training methodology that the HIIT group (training volume, intensity, frequency, type of exercise, training sessions and periodization) including electrical impulses with a whole-body electromyostimulation wireless device (Wiemspro®, Malaga, Spain), and following the manufacturer's instructions.

Electrical parameters

The periodization of electrical parameters for 12 weeks can be seen in Table 5. The HIIT+EMS periodization were designed considering different electrical parameters:

(i) *Frequency*: defined as the number of electrical pulses per time unit. Previous research has established that the ideal frequency to recruit type I fibers is 7–33 Hz [24]. Thus, the frequency applied in this program intervention was 15-33 Hz in the aerobic exercise (Type A session). On the other hand, the optimal frequency previously established to activate type II fibers is 35-100 Hz [24], so in the resistance exercises (type B session) the frequency applied was 35-75 Hz.

(ii) *Impulse width*: It could influence the intensity of muscle contraction and is specific for each muscle group. Their ranges from 200-400 µs according to scientific recommendations. In this sense, this parameter was adjusted in relation to the body

segment as follow: thigh zone (400 μs), glute zone (350 μs), abdominal zone (300 μs), dorsal zone (250 μs), cervical (200 μs), chest zone (200 μs), and arm zone (200 μs) [24].

(iii)   *Intensity:* defined as the percentage of maximum voluntary contraction. In this sense, the scientific guidelines established in local electrostimulation an intensity of >50 mA in order to improve body composition and fitness [24]. In this way the intensity applied in our intervention program was 80-100 mA depending on the type of session. The impulse intensity was individually adapted in each participant using the Borg CR-10 Scale "5" of "9" [25] in order to generate similar values of rate of perceived exertion (RPE) than other WB-EMS studies [12,19,26–28].

(iv)   *Duty cycle (stimulation ratio):* defined as the ratio between time receiving electrical stimuli and the total cycle time:

$$\% \text{ Duty cycle} = \frac{100}{\text{Total time/On-time}}$$

In relation to the frequency selected, a high duty cycle needs to be used with low frequencies to be feasible. Thus, A duty cycle of 50 – 63% was used on resistance training following scientific evidence, and a duty cycle of 99% was used on aerobic training since the frequency was low and the work time was of 3 min maximum.

Taking this in mind, the electric pulse applied was bipolar, symmetrical, and rectangular, programmed with the following electrical parameters depending on the type of session:

-    *Type A session (aerobic training):* We applied a frequency of 15-20 Hz, an intensity of 100 mA, an impulse breadth of 200-400 μs, and a duty cycle of 99%.

-    *Type B session (resistance training):* We applied a frequency of 35-75 Hz, an intensity of 80 mA, an impulse breadth of 200-400 μs, and a duty cycle of 50-63%.

Table 5. Electrical parameters in the HIIT+EMS periodization for 12 weeks

	FAMILIARIZATION PHASE					
Week	**1**		**2**		**3**	
Session (Type)	1 (A)	2 (B)	3 (A)	4 (B)	5 (A)	6 (B)
Frequency	15 Hz	35 Hz	15 Hz	35 Hz	15 Hz	40 Hz
Intensity	100 mA	80 mA	100 mA	80 mA	100 mA	80 mA
RPE impulse (0-10)	5-6	5-6	6-7	6-7	7-8	7-8
Duty cycle	99% (59¨:1¨)	50% (15¨:15¨)	99% (59¨:1¨)	57% (20¨:15¨)	99% (59¨:1¨)	50% (15¨:1
	PHASE I					
Week	**5**		**6**		**7**	
Session (Type)	9 (A)	10 (B)	11 (A)	12 (B)	13 (A)	14 (B)
Frequency	20 Hz	45 Hz	20 Hz	45 Hz	20 Hz	50 Hz
Intensity	100 mA	80 mA	100 mA	80 mA	100 mA	80 mA
RPE impulse (0-10)	7-8	7-8	7-8	7-8	7-8	7-8
Duty cycle	99% (59¨:1¨)	50% (15¨:15¨)	99% (59¨:1¨)	57% (20¨:15¨)	99% (59¨:1¨)	63% (25¨:1
	PHASE II					
Week	**9**		**10**		**11**	
Session (Type)	17 (A)	18 (B)	19 (A)	20 (B)	21 (A)	22 (B)
Frequency	20 Hz	60 Hz	20 Hz	65 Hz	20 Hz	70 Hz
Intensity	100 mA	80 mA	100 mA	80 mA	100 mA	80 mA
RPE impulse (0-10)	8-9	8-9	8-9	8-9	8-9	8-9
Duty cycle	99% (59¨:1¨)	50% (15¨:15¨)	99% (59¨:1¨)	57% (20¨:15¨)	99% (59¨:1¨)	63% (25¨:1

Type A: High intensity interval training on the treadmill with individual slope; Type B: High intensity interval training (weight-bear
RPE: Rating of perceived exertion.

A summary of the main characteristics of each training program is included in Table 6. All training sessions were supervised by a qualified and certified graduate in Sport Sciences, who constantly motivated the participants and instructed them to reach the specific target intensity in all sessions. Prior to each training session, all the participants underwent a dynamic standardized warm-up including general mobility exercises, and they concluded with a cooling-down protocol (active global stretching), alternating five posterior chain exercises with five anterior chain exercises. Heart rate was continuously monitored during exercise and the rated perceived exertion scale was registered in all sessions. Furthermore, a gradual progression was also proposed to control the exercise dose in each training group [1]. Attendance at the training sessions was registered daily. In this sense, to be included in the final analysis, participants were required to attend at least 90% of sessions. Finally, we provided no dietary prescription or instructions to the participants in the control and exercise groups.

CONTROL GROUP

The participants allocated to the non-exercise control group were asked to maintain their lifestyle (i.e., physical activity levels and dietary habits), and not being enrolled in any structured exercise program over the 12-week study period. Furthermore, for ethical reasons, the research staff invited to participants an informative meeting about a healthy lifestyle presided by graduates in Sport Sciences and Human Nutrition and Dietetics.

Table 6. Summary of the main components of exercise training programs

	PAR		HIIT		TYP... (LON...
Volumen	150 min/week at moderate intensity		40-65 min/ week at high intensity		
Intensity	**AEROBIC TRAINING**	**RESISTANCE TRAINING**	**TYPE A SESSION (LONG INTERVALS)**	**TYPE B SESSION (SHORT INTERVALS)**	**TYP... (LON...**
	60-65% of the HRres	40-50% of 1RM	>95% VO₂max	120% VO₂max (>90% of the HRres or <9 [0-10 RPE scale])	>9...
	Other variables were also considered: eccentric-isometric-concentric speed ratio, recovery time, and range of motion		The intensity (80-90% VO₂max) was progressively increased after the familiarization phase		The in... increas...
Frequency	• 3 days/week • Resistance training was performed on 2 of these 3 days/week • Rest from training of a minimum of 24 hours		• 2 days/week • 72-hour rest after a HIIT session		• •
Type of exercise	**AEROBIC TRAINING**	**RESISTANCE TRAINING**	**TYPE A SESSION (LONG INTERVALS)**	**TYPE B SESSION (SHORT INTERVALS)**	**TYP... (LON...**
	Treadmill, cycle-ergometer, and elliptical ergometer	weight–bearing and guided pneumatic machines, involving the major upper and lower body muscle groups	Walking on a treadmill with personalized slopes	8 weight-bearing exercise in circuit form: squat, dead lift, high knees up, high heels up, push up, horizontal row, lateral plank, and frontal plank	Walkin... with pe...
Electrical parameters					9...

PAR: Concurrent training based on physical activity recommendation from the World Health Organization; HIIT: High intensity interval training; HIIT+EMS electromyostimulation; HRres: Heart rate reserve; RM: Repetition maximum; VO₂max: Maximal oxygen uptake; RPE: Rating of perceived exertion; HZ: Hertz;

ASSESSMENT PERIOD

All baseline measurements were conducted between September and October of 2016/2017 at the *Centro de Investigación Deporte y Salud* (*CIDS*, Granada, Spain) and at the "Campus de la Salud" Hospital (Granada, Spain). The participants were due to attend the measurements on 4 different days. This assessment period was also conducted after the 12 weeks of the training intervention program. All measurements were supervised by experimental researchers of sport sciences, sport medicine and nutrition. Table 7 shows a summary of the different measurements carried out in every assessment day:

Table 7. Time distribution of the assessment period

Assessment day	Measured variables
Day 1	Medical examination (anamnesis, blood pressure, …) Blood sample collection *The measurements were conducted under fasting conditions*
Day 2	Anthropometry (weight, height, waist circumference, …) Body composition (dual energy X-ray absorptiometry scan) *The measurements were conducted under fasting conditions*
Day 3	Cardiorespiratory fitness (maximum exercise test on a treadmill) VO_2max (CPX Ultima CardiO2 metabolic cart) Electrocardiogram and blood pressure control
Day 4	Muscular strength Lower muscular strength (isokinetic dynamometry) Upper strength (isometric dynamometry)
Several days	Sedentary time and physical activity levels (triaxial accelerometry) Dietary intake (three 24 h recalls)

VO_2max: Maximal oxygen uptake.

OUTCOME MEASURES

Blood samples assessment (BSa)

The blood samples were obtained from the antecubital vein in the morning (8:30 AM – 10:00 AM) after an overnight fast and a minimum 10 min rest in a supine position. All samples were collected in prechilled Ethylenediamine tetra-acetic acid-containing tubes (Vacutainer, SST, Becton Dickinson, Plymouth, UK), and immediately centrifuged at four thousand revolutions per minute for seven minutes at 4ºC and stored at –80ºC until analysis.

$1,25(OH)_2D$ plasma levels were determined using a DiaSorin Liaison® immunochemiluminometric analyzer (DiaSorin Ltd, Wokingham, Berkshire, UK) and expressed as pg/mL. S-Klotho plasma levels were determined in ethylene diamine-acetic acid-treated plasma using a solid-phase sandwich enzyme-linked immunosorbent assay kit (Demeditec, Kiel, Germany) strictly following the manufacture's recommendations. The kit used two types of highly specific antibodies, and its optical density was measured at a wavelength of 450 nm ± 2 nm. The results were expressed in pg/mL.

We also measured other blood parameters including a general biochemical profile. In this sense, plasma glucose, triglycerides, total cholesterol, high density lipoprotein cholesterol (HDL-C), alanine transaminase (ALT), and γ-glutamyl transferase (γ-GT) were determined using an AU5800 absorption spectrophotometer (Beckman Coulter, Brea, CA, USA). Plasma insulin was assessed by chemiluminescence immunoassay using a UniCel DxI 800 paramagnetic particles (Beckman Coulter, Brea, CA, USA). Low density lipoprotein cholesterol (LDL-C) was calculated according to the following equation: LDL-C = (total cholesterol) – (HDL-C) – 0.45 x (triglycerides). Additionally, insulin/glucose, LDL-C/HDL-C, and triglycerides/HDL-C ratios were also calculated.

All participants were requested (i) to abstain from drugs and/or caffeine, (ii) to avoid any physical activity of moderate (24 hours before) and/or vigorous intensity (48 hours before), and (iii) to eat an established dinner before sampling (i.e., boiled rice, egg omelette, and tomato sauce). Blood samples were collected at the baseline and after 72–96 h of the last bout of exercise in the post-intervention assessment.

Anthropometric and body composition assessment (BCa)

Body weight and height were measured using a Seca model 799 scale and stadiometer (Seca, Hamburg, Germany) to the nearest 0.1 kg and 0.1 cm respectively, with participants wearing lightweight clothes and without shoes. Body mass index (BMI) was subsequently calculated as weight (kg)/height (m)^2. Waist circumference (WC) was assessed in a standing position from the mid-point between the bottom of the rib cage and the iliac crest after the end of a normal expiration. Body composition analysis was performed using a dual-energy X-ray absorptiometry scanner (Discovery Wi, Hologic, Inc., Bedford, MA, USA) obtaining lean body mass (kg), fat body mass (kg), and bone mineral content (BMC) (g). A spine phantom quality check scan was conducted on each study day. A whole-body scan was performed considering all manufacturer's guidelines (i.e., the positioning of participants, the analysis of results, and the quality controls among others). The APEX 4.0.2. software was used to draw an automatic delineation of anatomic regions. The lean mass index (LMI) was calculated as lean body mass (kg)/body height (m^2). Similarly, we calculated the fat mass index (FMI) as fat body mass (kg)/body height (m^2). Fat mass was also expressed as a percentage of the total body mass. Bone mineral density (BMD) was calculated as (bone mineral content [g]/ total bone surface [cm^2]).

Physical fitness assessment (PFa)

Cardiorespiratory fitness

A maximal treadmill (H/P/Cosmos Pulsar treadmill, H/P/Sport & Medical GMBH, Traumstein, Germany) exercise test was performed to determine maximal oxygen uptake (VO$_2$max) applying the modified Balke protocol [29]. Briefly, the test started with a brief warm-up walking at 3 km/h for 1 minute and at 4 km/h for 2 minutes. Thereafter, a speed of 5.3 km/h was fixed and progressive increments of grade (i.e., 1%) were established every minute until participants reached their volitional exhaustion. Gas exchange was registered by indirect calorimetry (CPX Ultima CardiO2, Medical Graphics Corp, St Paul, USA) using an oronasal mask (model 7400, Hans Rudolph Inc, Kansas City, MO, USA) equipped with a prevent™ metabolic flow sensor (Medgraphics Corp, Minnesota, USA). Flow calibration was performed using a 3-L calibration syringe before each test strictly following the manufacturer's instructions. The gas analyzer was calibrated before each test using two standard gas concentrations. O$_2$ consumption and CO$_2$ production were averaged each 5 seconds using the Breeze Suite software (version 8.1.0.54 SP7, MGC Diagnostic®). The 6-20 Borg scale rating of perceived exertion were applied during the last

15 seconds of each stage and at exhaustion [30]. We also registered heart rate (Polar RS300, Kempele, Finland) during the maximal treadmill exercise test every 5 seconds. The VO$_2$max criteria were defined as follow: (i) to attain a respiratory exchange ratio ≥ 1.1, (ii) to show a plateau in VO$_2$ (change of <100 ml/min in the last 30 seconds stage), (iii) to reach a maximal heart rate within 10 beats/min of the theoretical maximal heart rate (209-0.73 * age) [31]. Peak oxygen uptake value was considered when these criteria were not met [32].

The participants were previously instructed and asked about the following pre-conditions: (i) to refrain from stimulant substances at least 24 hours before the test, (ii) to fast for 3 hours and (iii) to avoid any physical activity of moderate and/or vigorous intensity for 24/48 hours before the test, respectively.

Muscular strength

The determination of muscular strength parameters was performed on a different day (separated by 72-96 hours) but within the same week under similar previous conditions than the cardiorespiratory fitness assessment.

An Isokinetic strength test was performed using a Gymnex Iso-2 dynamometer (Easytech s.r.l. Italy) applying the same preconditions as in the cardiorespiratory fitness measurement. The isokinetic dynamometer was calibrated following the manufacturer's instructions prior to starting the data collection. A concentric test of both knee flexor and extensor muscles was performed at an angular velocity of 60°/s. The participants were positioned on the seat, securing the upper leg, hips, and shoulder to the chair using safety belts, so as to avoid any movement of the body during the test. The limb was evaluated by positioning the lateral condyle of the femur in alignment with the rotational axis of the dynamometer, and the force pad was placed 3-4 cm above the medial malleolus. For safety reasons, a knee joint of motion angle between 90° and 170° was set for every participant. The participants were instructed to submaximally flex and extend the knee five times to become familiar with the test velocity and the movement required, followed by a 1-minute rest interval, then three maximal repetitions were completed in accordance with a previously validated protocol [33]. Extension and flexion peak torque were considered as the single repetition with the highest muscular force output (Nm). Constant verbal motivation encouragement was given to participants during the tests in order to generate maximum effort in each repetition, and the same trained researcher conducted all the isokinetic test.

A digital hand dynamometer (T.K.K. 5401 Grip-D; Takey, Tokyo, Japan) was used to determine hand grip strength (kg). Previously to measurements, one research team member demonstrated proper device and body positioning. Testing was performed two times on each

hand, alternately, with a 1-min interval between attempts, and, in each one, the participant was instructed to generate the greatest possible force during 2-3 s. Following previous studies, the optimal grip span of the dynamometer was adjusted at 5.5 cm for men, and calculated with a validated equation for women [34]. We considered total handgrip strength as the sum of the highest value (in kg) of each hand. The reported precision of the dynamometer was 0.1 kg.

Both cardiorespiratory fitness and muscular strength tests were conducted by the same research staff. VO_2max, extension and flexion peak torque, and hand grip strength were expressed in absolute values and in relation to weight and lean mass, respectively.

Physical activity assessment (PAa)

Triaxial accelerometers (GT3X+, Actigraph, Pensacola, FL, US) were used to objectively measured PA levels and sedentary time. They were placed in the participant's non-dominant wrist for 7 consecutive days, during 24h/day. The participants were instructed (i) to remove the accelerometers only during swimming and/or bathing activities and (ii) to make daily notes of their in-bed time. Raw accelerations were recorded with a frequency of 100 Hz and an epoch length of 5 seconds, and they were exported in .csv format using the software ActiLife v.6.13.3 (ActiGraph, Pensacola, FL, US). The GGIR package (v.1.6-0, https://cran.r-project.org/web/packages/GGIR/index.html) was subsequently used to process the raw data [35] in R (v.3.1.2, https://www.cran.r-project.org/). We used previously published methodologies to minimize the sensor calibration error (i.e., auto-calibration of the data based on local gravity) [36]. Accelerations were calculated by the Euclidean norm minus one (ENMO) as $\sqrt{(x^2 + y^2 + z^2)}$ - 1G (where 1G ~ 9.8 m/s^2). Sedentary time and PA outcomes (time spent per day in PA of light [LPA], moderate [MPA], vigorous [VPA], and moderate-vigorous [MVPA] intensity) were calculated through age-specific cut-points for ENMO [37,38]. Only data for participants who wore the accelerometers for more than 16 h/day and for at least 4 days including 1 weekend day, were included in final analyses.

Dietary intake assessment (DIa)

The participants were interviewed in person to obtain energy and macronutrients intake (i.e., fat, protein and carbohydrate) by the average of three 24-hours dietary recalls collected on non-consecutive days (2 days in middle of the week and 1 day on the weekend) which is a valid method to determine these parameters to within ~10% of the real energy intake [39]. The interviews were meal-sequence obtaining a detailed description of the food consumed by the participants.

The 24-hours dietary recalls were collected by a qualified and experimented research dietitian, using a colored-photographs guide to improve the quality of the information provided on portion sizes of food and to assists participants about the estimation of the food quantity consumed [40]. The software EvalFINUT® updated with databases from USDA (U.S. Department of Agriculture) and BEDCA ("Base de Datos Española de Composición de Alimentos") was used to calculate energy, macronutrients and micronutrients intake derived from the 24-hours recalls.

Cardiovascular disease risk assessment (CVDa)

A sex-specific cardiometabolic syndrome score (MetScore) was calculated for each participant based on the clinical guidelines proposed by the International Diabetes Federation according to the following factors: waist circumference, mean blood pressure, plasma glucose, HDL-C and triglycerides [41]. Standardized values were calculated for each variable as follows: Standardized values = (value – mean/standard deviation). The standardized HDL-C values were multiplied by -1 to indicate greater risk with higher values. MetScore was determined as the sum of these 5 standardized values divided by 5, to account for the number of variables included. This approach results in a continuous MetScore with a mean of 0 and a standard deviation of 1 by definition, considering lower values as a representation of a better cardiometabolic risk profile.

The Fatty liver index (FLI), a surrogate marker and validated algorithm of non-alcoholic fatty liver disease was calculated from BMI, waist circumference, triglycerides, and γ-GT according to a previously published report by Bedogni et al. [42]:

$$\text{FLI} = (e^{(0.953 \times \log e(\text{triglycerides}) + 0.139 \times \text{BMI} + 0.718 \times \log e(\gamma\text{-GT}) + 0.053 \times \text{waist circumference} - 15.745)}) \times 100$$

Quantitative insulin sensitivity check index (QUICKI) [43] was calculated from plasma insulin and glucose levels as:

$$\text{QUICKI} = 1/[\log(\text{plasma insulin (UI/mL)}) + \log(\text{plasma glucose (mg/dL)})]$$

The homoeostasis model assessment for insulin resistance index (HOMA-IR) [44] was calculated as:

$$\text{HOMA-IR} = \text{plasma insulin (UI/mL)} \times \text{plasma glucose (nmol/L)}/22.5$$

METHODOLOGICAL OVERVIEW OF THE STUDIES INCLUDED

The present International Doctoral Thesis is composed of a total of five studies. They are classified in two different sections: **Section 1** focuses on the relationship of 1,25-dihydroxyvitamin D with body composition, physical activity levels, physical fitness, cardiometabolic health and S-Klotho protein, (studies 1-4); and **Section 2** focuses on the effect of different exercise training modalities on 1,25-dihydroxyvitamin D (study 5). All studies contain data from the participants enrolled in the FIT-AGEING study. Table 8 shows an overview of the design, participants, and outcomes included in each study contained in the present International Doctoral Thesis.

Table 8. Methodological overview of the studies included in the present International Doctoral Thesis.

Study	General aim	Design	Participants
SECTION 1: 1,25-dihydroxyvitamin D and ageing markers: body composition, physical activity levels, physical fitness, Klotho protein			
Study 1	To study the relationship of 1,25(OH)$_2$D with body composition including lean and fat body mass as well as bone mineral density	Cross-sectional	73 middle-aged sedentary adults age 53.7 ± 5.1; BMI 26.7 ± 3.8), from
Study 2	To study the relationship of sedentary time, physical activity levels, and physical fitness (i.e., maximal oxygen uptake and muscular strength) with 1,25(OH)$_2$D	Cross-sectional	73 middle-aged sedentary adults age 53.7 ± 5.1; BMI 26.7 ± 3.8), from
Study 3	To study the relationship of 1,25(OH)$_2$D with cardiometabolic risk factors	Cross-sectional	73 middle-aged sedentary adults age 53.7 ± 5.1; BMI 26.7 ± 3.8), from
Study 4	To investigate the relationship between 1,25(OH)$_2$D and S-Klotho, and to study the mediation role of body composition in the relationship between 1,25(OH)$_2$D and S-Klotho	Cross-sectional	73 middle-aged sedentary adults age 53.7 ± 5.1; BMI 26.7 ± 3.8), from

SECTION 2: Role of exercise on 1,25-dihydroxyvitamin D

Study 5	To study the effects of 12 weeks different training modalities ([a] a concurrent training based on physical activity recommendation from the World Health Organization group, [b] a high-intensity interval training group, and [c] a high-intensity interval training group adding whole-body electromyostimulation group) on 1,25(OH)$_2$D as well as to examine whether these hypothetical changes in 1,25(OH)$_2$D are associated with changes in body composition and physical fitness	Randomized controlled trial	- Non-exercise group (n= 15 ~60% w ± 4.1; BMI 26.7 ± 3.9) - PAR group (n= 53~% women; age 25.4 ± 2.9) - HIIT group (n= 16 ~56% women BMI 26.1 ± 3.2) - HIIT+EMS group (n= 18 ~50% wo 5.3; BMI 28.5 ± 4.7)

1,25(OH)$_2$D: 1,25-dihydroxyvitamin D; S-Klotho: Shed form of the Klotho protein; BSa: Blood samples assessment; BCa: Anthropom PAa: Physical activity assessment; DIa: Dietary intake assessment; PFa: Physical fitness assessment; CVDa: Cardiovascular disease ris based on physical activity recommendation from the World Health Organization; HIIT: High intensity interval training; HIIT+EMS: whole-body electromyostimulation.

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RESULTS AND DISCUSSION

SECTION 1

1,25-dihydroxyvitamin D and ageing markers: body composition, physical activity levels, physical fitness, cardiometabolic health, and S-Klotho protein

Chapter 3

Relationship between 1,25-dihydroxyvitamin D and body composition in middle-aged sedentary adults:

the FIT-AGEING Study **(Study 1)**

ABSTRACT

Background & aims: Vitamin D deficiency is a worldwide health problem that, in addition to its well-known negative effects on musculoskeletal health, has been related to a wide range of acute and chronic age-related diseases. However, little is known about the association of body composition with the active, hormonal form of vitamin D, 1,25-dihydroxyvitamin D plasma levels (1,25(OH)$_2$D). Therefore, the aim of this study was to investigate the association of 1,25(OH)$_2$D with body composition including lean and fat body mass as well as bone mineral density (BMD) in middle-aged sedentary adults.

Methods: A total of 73 (39 women) middle-aged sedentary adults (53.7 ± 5.1 years old) participated in the current study. We measured weight and height, and we used dual energy X-ray absorptiometry to measure lean body mass, fat body mass and BMD. Body mass index (BMI), lean mass index (LMI), and fat mass index (FMI) were calculated. 1,25(OH)$_2$D was measured using a DiaSorin Liaison®immunochemiluminometric analyzer.

Results: The results showed a negative association of 1,25(OH)$_2$D with BMI, LMI and BMD (β=−0.274, R^2=0.075, P=0.019; β=−0.268, R^2=0.072, P=0.022; and β=−0.325, R^2=0.105, P=0.005, respectively), which persisted after controlling for age and sex. No significant differences in 1,25(OH)$_2$D across body weight status were observed after controlling for the same covariates.

Conclusions: Our results suggest that 1,25(OH)$_2$D could be negatively associated with BMI, LMI and BMD whereas no association was found with FMI in middle-aged sedentary adults.

BACKGROUND

As the world´s population ages, the prevalence of chronic diseases increases, particularly over the last decades, becoming one of the great challenges that society faces [1,2]. Abnormalities in body composition such as a decrease of lean body mass and/or bone mineral density (BMD) or an increment in fat body mass are powerful predictors of morbidity and mortality risk as well as overall quality of life [3]. Epidemiologic studies indicate that these body composition changes are closely related to obesity, sarcopenia and/or osteoporosis in the elderly population [3,4]. However, these chronic diseases are progressive and initiate at a younger age [5,6]. Their high prevalence and concomitant health risk make them a particularly relevant worldwide public health problem and a social and economic burden [7–9].

Vitamin D is a fat-soluble vitamin essential for normal homeostasis of calcium and phosphorus, as well as for bone health [10] and preventing falls [11] and fractures [12]. Globally, vitamin D deficiency has been considered as a major public health problem affecting not only musculoskeletal health but also a wide range of several age-related chronic diseases [13]. 25-hydroxyvitamin D (25(OH)D) is the most commonly used biomarker when evaluating the relationship of vitamin D status with health-related outcomes [14–17]. However, 25(OH)D is thought to be largely inactive since it requires to be metabolized in the kidney by the enzyme 25-hydroxyvitamin D-1α-hydroxylase to be active. Therefore, 1,25-dihydroxyvitamin D (1,25(OH)$_2$D), also known as calcitriol, is responsible for most, if not all, of its biological effects [18,19]. The association between 1,25(OH)$_2$D and body composition parameters has been hard to establish for several reasons: (i) previously there was not a reliable and sensitive assay for calcitriol [20,21] and (ii) 25(OH)D has a higher concentration and longer half-life than 1,25(OH)$_2$D, thus requiring less sample volume for reliable measurements [22].

Consequently, epidemiological data are scarce, and a review of the scientific literature found no large studies examining the relationship between 1,25(OH)$_2$D and body composition outcomes. There is some evidence of the existence of an inverse association of 1,25(OH)$_2$D with body mass index (BMI) and fat body mass [23,24], however these data are conflicting with other studies [25–28]. In contrast, data are scarce on the potential relationship between 1,25(OH)$_2$D and lean body mass. Two recent studies found a significant association between low 1,25(OH)$_2$D and low lean body mass [29,30], which is highly dependent on the individual's age [29]. Similarly, controversial findings have been discovered regarding the role of 1,25(OH)$_2$D in bone health. Previous cross-sectional studies have reported an inverse association between 1,25(OH)$_2$D and

BMD [31–33], whereas other studies found that 1,25(OH)$_2$D was unrelated to bone mineral content [34], bone loss [33,35] or hip fracture risk [36,37].

There are limited data on the study of body composition parameters in relation to 1,25(OH)$_2$D status in middle-aged adults. Thus, understanding whether 1,25(OH)$_2$D is associated with body composition parameters in this population is of clinical interest since, as previously established, the interventions to delay or reverse body composition related diseases are preferable when individuals are still relatively young and healthy [38,39].

Therefore, the aim of this study was to investigate the association of 1,25(OH)$_2$D with body composition including lean and fat body mass as well as BMD in middle-aged sedentary adults.

MATERIALS AND METHODS

Study design and participants

The present cross-sectional study was conducted under the framework of the FIT-AGEING study (clinicaltrial.gov: ID: NCT03334357) [40]. The Ethics Committee on Human Research of the Regional Government of Andalucía approved the rationale, design, and methodology of the study [0838-N-2017] and all participants signed written informed consent in accordance with the Declaration of Helsinki (last revision guidelines, 2013). Seventy-three middle-aged sedentary adults were recruited via electronic media, social networks and leaflets. The inclusion criteria were as follows: (i) to be sedentary (i.e., less than 20 min of physical activity on less than 3 days/week), (ii) not to have had greater body weight changes than 3 kg in the past 3 months, (iii) to be aged between 45 and 65 years old, (iv) not to be a smoker, (v) to be taking no long-term medication, (vi) not to be pregnant and (vii) not to suffer from any chronic cardiometabolic disease.

All tests were performed during September–October 2016/17 at the *Centro de Investigación Deporte y Salud* (*CIDS*, Granada, Spain) and at the "Campus de la Salud" Hospital (Granada, Spain).

Anthropometric parameters and body composition assessment

A pre-validated Seca model 799 scale and stadiometer (Seca, Hamburg, Germany) was used to measure body weight and height with light clothing and without shoes. BMI was subsequently calculated as weight (kg)/height (m^2). Body composition outcomes were determined by a dual-energy X-ray absorptiometry scanner (DXA, Discovery Wi, Hologic, Inc.,

Bedford, MA, USA) obtaining lean body mass in kg, fat body mass in kg, and BMD in g/cm2. A spine phantom quality check scan was conducted on each study day. A whole-body scan was performed considering all manufacturer′s guidelines (i.e., the positioning of participants, the analysis of results and the quality controls among others). The APEX 4.0.2. software was used to draw an automatic delineation of anatomic regions. The lean mass index (LMI) was calculated as lean body mass (kg)/body height (m²). Similarly, we calculated the fat mass index (FMI) as fat body mass (kg)/body height (m²). Fat mass was also expressed as a percentage of the total body mass. The participants were categorized into three groups on the basis of BMI levels: (i) normal weight (BMI ≥ 18.5 and < 25 kg/m²), (ii) overweight (BMI ≥ 25 and <30 kg/m²), and (iii) obese (BMI ≥ 30 kg/m²).

Dietary intake assessment

We performed a total of three 24-hour dietary recalls collected on non-consecutive days (one weekend day included). This validated method is able to determine the energy intake to within 8–10% of the current energy intake [41]. The interviews were meal sequence-based, in which a detailed description of the food consumed by the participants was recorded. The 24-hour dietary recalls were collected by an experimented and qualified research dietitian, using a photograph guide to improve the quality of the information provided on portion sizes of food and assisting participants in the estimation of the consumed food quantity [42]. The software EvalFINUT® updated with data from USDA (U.S. Department of Agriculture) and BEDCA ("Base de Datos Española de Composición de Alimentos") was used to calculate energy and micronutrient (i.e., vitamin D, calcium and phosphorus) intake derived from the 24-hour recalls.

Physical activity assessment physical

Physical activity levels were objectively assessed with a wrist-worn accelerometer (ActiGraph GT3X+, Pensacola, FL, United States) for 7 consecutive days (24 hours/day) [40]. The sampling frequency was previously set at 100 Hz to store raw accelerations [43]. The ActiLife v.6.13.3 software (ActiGraph, Pensacola, FL, United States) and the GGIR package (v.1.5-12) in R (v.3.1.2) were used to process these files [44,45]. The participants came to the laboratory and specific information about how to wear the accelerometer was given. They were also reminded to remove it only during water-based activities such as swimming or bathing. Only the participants who wore the accelerometer for ≥16 hours/day for 4 days (including 1 weekend day) were included in the analysis.

Blood samples assessment

A 10 mL peripheral blood sample was taken from the antecubital vein after overnight fasting. It was collected using the Vacutainer SST system (Becton Dickinson, Plymouth, UK) in ethylenediamine tetra-acetic acid-containing tubes. Blood samples were centrifuged at four thousand revolution per minute for seven minutes at 4 ∘C and stored at −80 ∘C. Plasma levels of 1,25(OH)$_2$D were measured using a DiaSorin Liaison®immunochemiluminometric analyzer (DiaSorin Ltd, Wokingham, Berkshire, UK) and expressed in pg/mL.

Statistical analysis data

Data were checked for normality with the use of distribution plots (i.e., visual check of histograms, and Q-Q plots) and the Shapiro-Wilk test. The descriptive parameters were reported as mean and standard deviation.

Differences between sexes were examined using an independent samples T test. Given that no interaction for sex was observed ($P>0.05$), data are presented for men and women together. Simple linear regression models were built to test the association of 1,25(OH)$_2$D and body composition outcomes (i.e., BMI, LMI, FMI, and BMD). We also performed multiple linear regression models to analyze these associations controlling for age (Model 1), sex (Model 2), and age and sex (Model 3). Additionally, we also adjusted these models for total energy, vitamin D, calcium, phosphorus intake and/or physical activity levels (i.e., light, moderate-vigorous and total physical activity). To test whether 1,25(OH)$_2$D was different across body weight status (i.e., normal-weight, overweight and obese individuals), an analysis of variance (ANOVA) was conducted. Moreover, we performed an analysis of covariance to test the differences of 1,25(OH)$_2$D across weight status adjusting for age and sex.

Data were analyzed with the use of the Statistical Package for Social Sciences (SPSS, v. 22.0, IBM SPSS Statistics, IBM Corporation, Armonk, NY, USA). Graphical plots were built using the GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The level of significance was fixed at <0.05.

RESULTS

Table 1 shows the descriptive parameters of our study participants by sex. No significant differences in 1,25(OH)$_2$D were observed between men and women (P=0.576).

Table 1. Descriptive characteristics of participants.

	N	All		N	Men		N	Women	
Age (years)	73	53.7	(5.1)	34	54.6	(5.2)	39	53	(5.0)
Body composition parameters									
Body mass index (kg/m²)	73	26.7	(3.8)	34	28.3	(3.6)	39	25.3	(3.3) *
Lean mass (kg)	73	43.2	(11.7)	34	53.9	(6.5)	39	34.1	(5.8) *
Lean mass index (kg/m²)	73	15.2	(2.9)	34	17.5	(2.0)	39	13.2	(1.8) *
Fat mass (%)	73	40.1	(8.9)	34	34.7	(8.0)	39	44.5	(7.4) *
Fat mass (kg)	73	30.1	(8.5)	34	30.9	(9.8)	39	29.2	(7.1)
Fat mass index (kg/m²)	73	10.8	(3.1)	34	10.0	(3.2)	39	11.4	(2.9)
Bone mineral density (g/cm²)	73	1.1	(0.1)	34	1.2	(0.1)	39	1.0	(0.1) *
Dietary intake									
Total Energy intake (kcal/day)	72	2071.7	(455.4)	34	2312.1	(402.9)	38	1854.6	(390.3) *
Vitamin D intake (µg/day)	72	5.0	(6.0)	34	3.8	(3.3)	38	6.1	(7.6)
Calcium intake (mg/day)	72	763.4	(340.5)	34	867.3	(396.9)	38	670.5	(251.4) *
Phosphorus intake (mg/day)	72	1324.7	(558.9)	34	1507.6	(689.6)	38	1161.0	(342.2) *
Physical activity parameters									
LPA (min/day)	70	173.7	(45.4)	33	169.9	(52.7)	37	178.0	(40.7)
MVPA (min/day)	70	95.8	(35.6)	33	96.4	(37.1)	37	96.6	(35.7)
Total PA (min/day)	70	269.5	(75.1)	33	265.2	(79.3)	37	273.3	(72.0)
Blood parameters									
1,25(OH)$_2$D (pg/ml)	73	40.3	(14.1)	34	38.3	(13.4)	39	42.0	(14.6)

Dara are presented as means (standard deviation). * Significance differences between sexes (P<0.05) obtained by the independent sample T test. LPA: Light physical activity; MVPA: Moderate-vigorous physical activity; PA: Physical activity; 1,25(OH)$_2$D: 1,25-dihydroxyvitamin D.

Figure 1 shows the associations between 1,25(OH)$_2$D and body composition related parameters. There was a significant negative association of 1,25(OH)$_2$D with BMI (β=−0.274, R²=0.075, P=0.019, Figure 1A), LMI (β=−0.268, R²=0.072, P=0.022, Figure 1B) and BMD (β=−0.325, R²=0.105, P=0.005, Figure 1D), which persisted after including age, sex, and age and sex in the model (all P≤0.042, Table 2). 1,25(OH)$_2$D was not significantly associated with FMI (β=−0.080, R²=0.006, P=0.502; Figure 1C), which did not change adjusting for age, sex, and age and sex (all P≥0.35, Table 2). The results remained unchanged after further adjusting for total energy, vitamin D, calcium, phosphorus intake and/or physical activity levels (i.e., light, moderate-vigorous and total physical activity) (data not shown, all P>0.1).
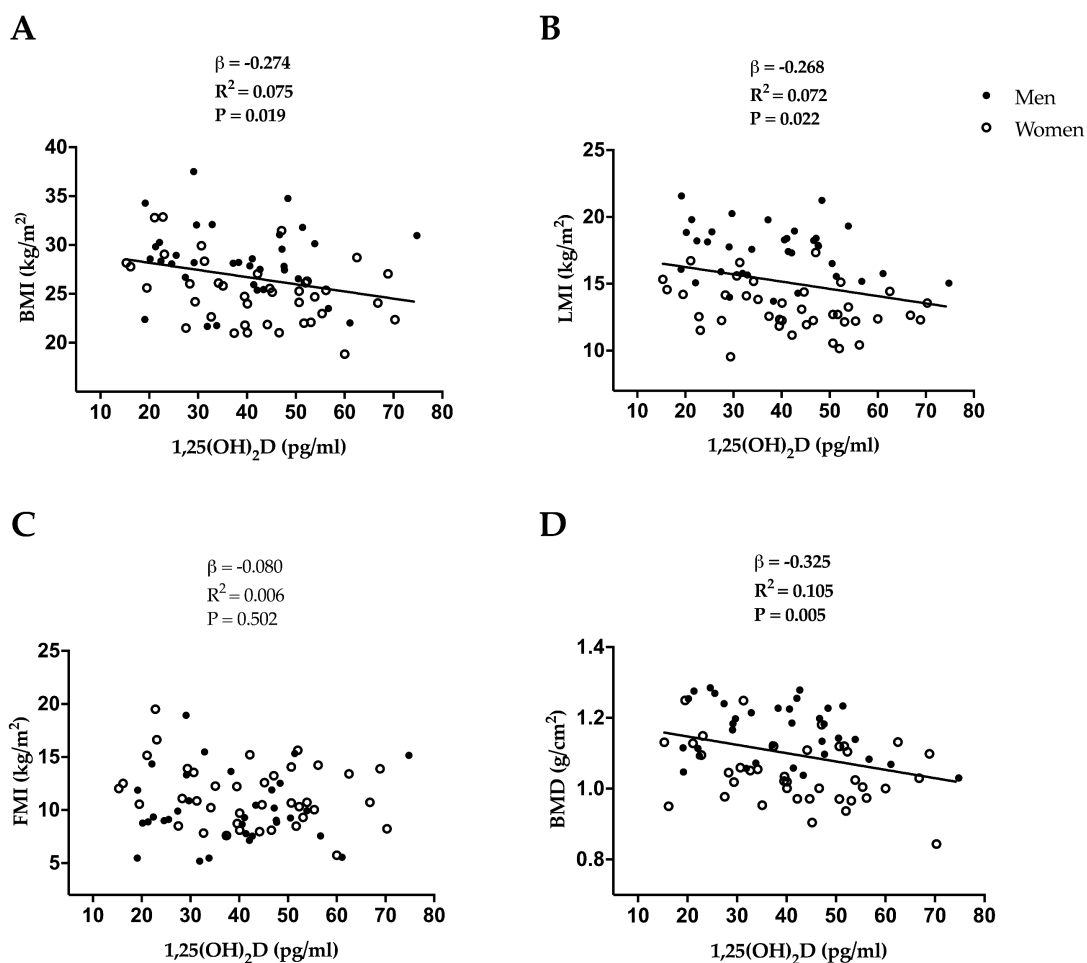
Figure 1. Simple linear regression graphs between 1,25-dihydroxyvitamin D (1,25(OH)2D) and body mass index (BMI, Figure 1A), lean mass index (LMI, Figure 1B), fat mass index (FMI, Figure 1C), and bone mineral density (BMD, Figure 1D) in middle-aged sedentary adults. β (standardized regression coefficient), R^2, and P from a simple linear regression analysis.

Table 2. Association of 1,25-dihydroxyvitamin D with body mass index, lean mass index, fat mass index and bone mineral density.

	1,25-dihydroxyvitamin D					
	Model 1		Model 2		Model 3	
	P value	β	P value	β	P value	β
Body mass index (kg/m²)	**0.020**	−0.274	**0.040**	−0.263	**0.042**	−0.262
Lean mass index (kg/m²)	**0.023**	−0.269	**0.030**	−0.383	**0.032**	−0.383
Fat mass index (kg/m²)	0.505	−0.080	0.354	−0.112	0.356	−0.113
Bone mineral density (g/cm²)	**0.005**	−0.325	**0.009**	−0.370	**0.009**	−0.377

Model 1 was adjusted for age; Model 2 was adjusted for sex; and Model 3 was adjusted for age and sex. P value of multiple-regression analysis. β (standardized regression coefficient). Values in bold indicate significance differences (P<0.05).

ANOVA revealed no significant differences in 1,25(OH)$_2$D across body weight status (45.3 ± 13.3 pg/mL in normal-weight, 37.8 ± 13.1 pg/mL in overweight and 38.4 ± 16.7 pg/mL in obese individuals; Figure 2), which persisted after including age and sex as a covariate (44.8 ± 13.1 pg/mL in normal-weight; 37.9 ± 12.3 pg/mL in overweight and 38.9 ± 14.0 in obese individuals; P=0.2).
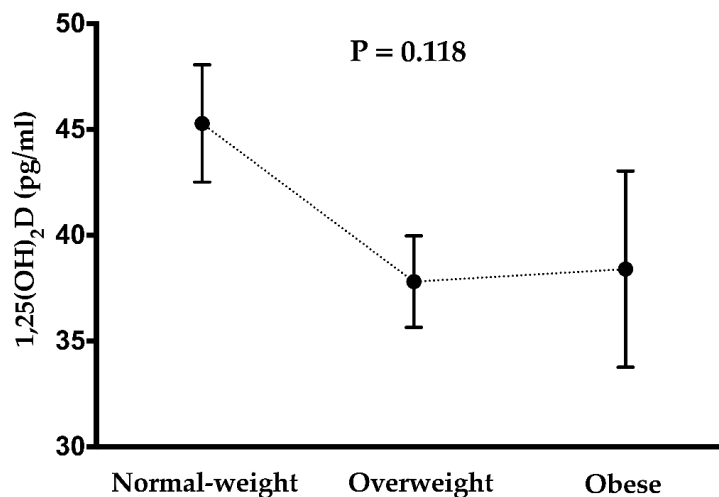
Figure 2. 1,25-dihydroxyvitamin D (1,25(OH)$_2$D) by body weight status categories in middle-aged adults. Values are presented as means and standard error. P value obtained from the analysis of the variance to compare 1,25(OH)$_2$D across weight status (normal-weight, over-weight, and obese).

DISCUSSION

The main results of the present study suggest that 1,25(OH)$_2$D is negatively associated with BMI, LMI and BMD independently of age and sex, whereas no association was found between 1,25(OH)$_2$D and FMI in middle-aged sedentary adults.

There is a controversy in the scientific literature regarding the 1,25(OH)$_2$D status of obese individuals. It has been described in classical studies that there are greater levels of 1,25(OH)$_2$D (~20 to 30%) in both obese men and women [25–28,46]. However, although we did not find any significant differences between BMI groups, we observed that overweight and obese individuals have ~16.6% and ~15.3% lower 1,25(OH)$_2$D than normal-weight individuals, which agrees with a previous large cohort that reported a ~18% lower 1,25(OH)$_2$D in the obese group compared with their lean counterparts [23,24]. These discrepancies between studies cannot be attributed to the individual′s age, sex and/or BMI, since the participants had similar biological characteristics. A seasonal effect might explain these controversial results as the 1,25(OH)$_2$D assessment was conducted in winter months [46] or spring months [27] in some studies, while others did not control

the season in which the blood samples were taken [24]. Given that the effects of ultraviolet ray exposure on 25(OH)D could be different for obese and lean individuals, the time of the year when the study was conducted may be of importance. However, previous studies have suggested that the season of the year does not present the same influence on 1,25(OH)$_2$D as it has on 25(OH)D [47]. The different 1,25(OH)$_2$D assay methods used across the studies could be one important factor to consider. While older studies applied radioreceptor assays [25–28,46] in which lipid interferences have been described when samples are not pure enough [48], more recent studies including our own study used modern immuno assays for the assessment of 1,25(OH)$_2$D [30,32]. Taken all together, it is likely that obese subjects present low 1,25(OH)$_2$D. This fact could be explained because obesity is associated with poor levels of 25(OH)D, and it has been demonstrated that 1,25(OH)$_2$D depends on substrate availability [23,49]. A higher fat body mass offers a greater distribution space for both fat-soluble compounds. Additionally, obese individuals are usually exposed to negative lifestyle factors (i.e., unhealthy dietary habits and sedentary behaviour, among others), [50] and to lower sunlight exposure [51]. These lifestyle factors have been shown to have a negative influence on vitamin D status [52,53].

The use of 25(OH)D as a key marker of vitamin D status has logical advantages summarized as greater serum stability and a longer half-life than other markers (e.g., 1,25(OH)$_2$D) [22]. However, it is important to consider that it is biologically inactive and therefore could not be the best vitamin D function indicator [54]. The shorter half-life of 1,25(OH)$_2$D could be one of the reasons why the relationship of 1,25(OH)$_2$D and lean body mass has not been deeply studied. Hassan-Smith et al. reported a positive association between 1,25(OH)$_2$D and lean body mass [29]. These findings differ from those observed in our study in which we obtained higher 1,25(OH)$_2$D in individuals with lower lean body mass. These discrepancies could be explained by the different biological characteristics of the subjects of the study (i.e., our cohort was older than the Hassan-Smith et al. study [29]) and by the different assay methods used to determine 1,25(OH)$_2$D. New studies measuring 1,25(OH)$_2$D are necessary to clarify this issue.

We also found a negative association between 1,25(OH)$_2$D and BMD in our study cohort which concurs with a previous study that reported a positive association of 1,25(OH)$_2$D and the bone resorption marker β-cTX [32]. These results are consistent with the notion that 1,25(OH)$_2$D increases bone resorption via stimulating intestinal calcium absorption after calcium intake [32]. Moreover, recent animal and in vitro studies have proposed that 1,25(OH)$_2$D has a direct effect on osteoclasts inducing bone resorption by its interaction with the receptor activator of nuclear factor-κβ/receptor activator of nuclear factor-κβ ligand signaling pathway [55,56]. Taking this into consideration, an inverse association would be expected between 1,25(OH)$_2$D and bone mineral

density. In addition, it seems plausible that $1,25(OH)_2D$ is produced when BMD is low and once BMD is recovered $25(OH)D$ is transformed into the inactive $24,25(OH)_2D$. Although this argument could explain our findings, further intervention studies are needed to understand this issue.

Limitations

Our study has some limitations. The cross-sectional design does not allow ascribing causality to the observed relationships. Further, our study population was limited to sedentary healthy middle-aged adults (45–65 years old) and hence these results may not be generalizable to younger, older, and/or physically active individuals. This study, like most clinical studies, was based on a single assay of $1,25(OH)_2D$. We did not assess the 24-hydroxyvitamin D_2 plasma levels. In addition, a whole-body DXA scan was conducted, so future studies are necessary to investigate whether spine and hip bone mineral density have the same association pattern. Finally, due to the relatively small sample size of the current study, the data should be interpreted with caution. One of the strengths of this study is that body composition was measured using a gold-standard technology, such as dual-energy X-ray absorptiometry. In addition, the measurement of objective physical activity data and dietary intake to be used as covariates represent further strengths.

CONCLUSIONS

In conclusion, our results suggest that $1,25(OH)_2D$ could be negatively associated with BMI, LMI and BMD independently of age and sex, while no significant relationship was obtained between $1,25(OH)_2D$ and FMI in middle-aged sedentary adults. Intervention studies are needed to understand whether changes in body composition status are associated with changes in $1,25(OH)_2D$ in this age-population.

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Chapter 4

Relationship of sedentary time, physical activity levels, and physical fitness with 1,25-dihydroxyvitamin D in middle-aged sedentary adults: the FIT-AGEING study **(Study 2)**

ABSTRACT

Background & aims: Potential interactions between sedentary behaviour, physical activity (PA), and physical fitness with 25-hydroxyvitamin D (25(OH)D) status have been previously suggested. However, data are scarce concerning the association between with these predictors of general health and the main active metabolite of vitamin D, the 1,25-dihydroxyvitamin D (1,25(OH)$_2$D). Therefore, the aim of this study was to analyse the relationship of sedentary time, PA levels, and physical fitness (i.e., maximal oxygen uptake (VO$_2$max) and muscular strength) with 1,25(OH)$_2$D in middle-aged sedentary adults.

Methods: A total of 73 (39 women) middle-aged sedentary adults (53.7 ± 5.1 years old) participated in this cross-sectional study. Sedentary time and PA intensity levels were objectively measured with triaxial accelerometers for 7 consecutive days. VO$_2$max was determined by a maximum treadmill test employing indirect calorimetry. Lower and upper limb muscular strength was assessed by an isokinetic strength test and by a handgrip strength test, respectively. 1,25(OH)$_2$D plasma levels were measured using a DiaSorin Liaison® immunochemiluminometric analyzer.

Results: No significant relationships were found between objectively measured sedentary time, PA levels or physical fitness (i.e., VO$_2$max, extension and flexion peak torque, and hand grip strength) and 1,25(OH)$_2$D (all P>0.05). All results persisted after controlling for age, sex, fat mass or energy, vitamin D, calcium, and phosphorus intake.

Conclusions: In summary, our results suggest that sedentary time, PA intensity levels, and physical fitness are not associated with 1,25(OH)$_2$D in middle-aged sedentary adults.

BACKGROUND

Physical inactivity and high levels of sedentary behaviour are currently recognized as a serious public health problem [1] being both independently associated with poorer life quality, lower life expectancy, higher incidence of chronic diseases, and an increased risk of all-cause mortality [2–5]. Physical exercise (i.e., programmed physical activity (PA)) is considered a highly effective strategy to improve health-related outcomes through physical fitness improvements [6,7]. Previous studies have reported that cardiorespiratory fitness and muscular strength -as the main components of physical fitness- are important predictors of cardiometabolic risk [8], all-cause mortality [9–11], and life expectancy [12,13]. It is therefore of scientific interest to identify factors that explain the relationships of sedentary time, PA levels, and physical fitness with general health during adulthood to well-understand the physio-pathological mechanisms that occur during the ageing process.

Vitamin D status has received considerable attention in a clinical context during the last years given its multiple physiological functions [14,15]. In addition to its key role in calcium homeostasis maintenance and bone metabolism, receptors for vitamin D can be found in at least thirty different types of cells -including skeletal muscle cells [16]- potentially exerting additional metabolic functions. Indeed, several studies have suggested that low levels of vitamin D are associated with numerous non-skeletal disorders, such as cardiovascular diseases [17–19], certain types of cancers [20], and others age-related diseases [21].

It has been shown that higher PA levels are associated with higher vitamin D levels in different populations [22–27], but there are controversial findings regarding the relationship of sedentary behaviour and vitamin D levels [22,25,28]. Vitamin D status has been usually defined as serum 25-hydroxyvitamin D levels [25(OH)D]. However, limited studies have assessed the main active metabolite: the 1,25-dihydroxyvitamin D [1,25(OH)$_2$D], also known as calcitriol, that is responsible for the majority of its biological effects [29,30].

In this context, there are only a few recent studies investigating the associations of PA levels and sedentary behaviour with 1,25(OH)$_2$D in both elderly men [31] and patients with colorectal adenoma [25] obtaining contradictory results. These discrepancies could be explained by the use of subjective questionnaires to assess PA levels and sedentary behaviour with their consequent bias, and the pathological status of the patients with colorectal adenoma as well. Moreover, it has been reported a modest positive association between 25(OH)D and cardiorespiratory fitness [32]. However, to date, there are no studies exploring the relationship

between cardiorespiratory fitness and 1,25(OH)$_2$D while controversial evidence has been observed regarding the association of muscular strength and 25(OH) [33–38].

The relationship of sedentary time, PA levels, and physical fitness with 1,25(OH)$_2$D levels has been challenging to establish due to different causes: [i] 25(OH)D has a longer half-life and higher concentration than 1,25(OH)$_2$D requiring less sample volume for a reliable measurement [39,40], and [ii] until recently, there has not been a sensitive and reliable assay for 1,25(OH)$_2$D assessment [41]. Thus, analysing the association of the above-mentioned predictors of general health (all of them measured with gold standard methods) with 1,25(OH)$_2$D is of clinical interest given that 1,25(OH)$_2$D is the most metabolically active form of vitamin D. Therefore, this study aimed to analyse the relationship of sedentary time, PA levels, and physical fitness with 1,25(OH)$_2$D in middle-aged sedentary adults.

MATERIALS AND METHODS

Study design and participants

This cross-sectional study was conducted under the framework of the FIT-AGEING study (clinicaltrial.gov: ID: NCT03334357), an exercise-based randomized controlled trial [42]. Seventy-three middle-aged sedentary adults (52% women) were included in the present study. Participants were recruited via social networks, electronic media, and leaflets advertising. The evaluation tests were conducted between September-October 2016 and 2017 at the *Centro de Investigación Deporte y Salud (CIDS,* Granada, Spain*)* and at the "Campus de la Salud" Hospital (Granada, Spain). The inclusion criteria were to be sedentary (defined as practicing <20 min of PA on <3 days per week [self-reported]), to be aged 45-65 years old, to be taking no drugs or long-term medication, not to be a smoker, to not suffer from any cardiometabolic illness, to not be pregnant and to not have important weight changes (<5 kg) during the last 3 months.

This study was designed and conducted strictly following the latest revision of the declaration of Helsinki (2013) and all participants gave their written and verbal informed consent. The study was approved by the Ethics Committee on Human Research of the Regional Government of Andalucía [0838-N-2017].

Anthropometric measurements and body composition assessment

Body weight and height were measured using a Seca model 799 scale and stadiometer (Seca, Hamburg, Germany) to the nearest 0.1 kg and 0.1 cm respectively, with participants wearing

lightweight clothes and barefoot. Body mass index (BMI) was then calculated as weight (kg)/height (m)^2. Body composition analysis was performed using a dual-energy X-ray absorptiometer scanner (Discovery Wi, Hologic, Inc., Bedford, MA, USA), obtaining lean and fat body mass in kg following the manufacture´s recommendations and fat mass percentage was calculated.

Sedentary time and physical activity levels assessment

Triaxial accelerometers (GT3X+, Actigraph, Pensacola, FL, US) were used to objectively measured sedentary time and PA levels. They were placed in the participant´s non-dominant wrist for 7 consecutive days, during 24h/day. The participants were instructed (i) to remove the accelerometers only during swimming and/or bathing activities and (ii) to register daily notes of their in-bed time. Raw accelerations were recorded with a frequency of 100 Hz and an epoch length of 5 seconds, and they were exported in .csv format using the software ActiLife v.6.13.3 (ActiGraph, Pensacola, FL, US). The GGIR package (v.1.6-0, https://cran.r-project.org/web/packages/GGIR/index.html) was subsequently used to process the raw data [43] in R (v.3.1.2, https://www.cran.r-project.org/). We used previously published methodologies to minimize the sensor calibration error (i.e., auto-calibration of the data based on local gravity) [44]. Accelerations were calculated by the Euclidean norm minus one (ENMO) as $\sqrt{(x^2 + y^2 + z^2)} - 1G$ (where $1G \sim 9.8$ m/s^2). Sedentary time and PA outcomes (time spent per day in PA of light [LPA], moderate [MPA], vigorous [VPA], and moderate-vigorous [MVPA] intensity) were calculated through age-specific cut-points for ENMO [45,46]. Only data for participants who wore the accelerometers for more than 16 h/day and at least 4 days including 1 weekend day, were included in the final analyses.

Cardiorespiratory fitness assessment

A maximal treadmill (H/P/Cosmos Pulsar treadmill, H/P/Sport & Medical GMBH, Traumstein, Germany) exercise test was performed to determine maximal oxygen uptake (VO$_2$max) applying the modified Balke protocol [47]. Briefly, the test started with a brief warm-up walking at 3 km/h for 1 minute and at 4 km/h for 2 minutes. Thereafter, a speed of 5.3 km/h was fixed and progressive increments of grade (i.e., 1%) were established every minute until participants reached their volitional exhaustion. Gas exchange was registered by indirect calorimetry (CPX Ultima CardiO2, Medical Graphics Corp, St Paul, USA) using an oronasal mask (model 7400, Hans Rudolph Inc, Kansas City, MO, USA) equipped with a prevent™ metabolic flow sensor

(Medgraphics Corp, Minnesota, USA). Flow calibration was performed using a 3-L calibration syringe before each test strictly following the manufacturer's instructions. The gas analyzer was calibrated before each test using two standard gas concentrations. O_2 consumption and CO_2 production were averaged every 5 seconds using the Breeze Suite software (version 8.1.0.54 SP7, MGC Diagnostic®). We also registered heart rate (Polar RS300, Kempele, Finland) during the maximal treadmill exercise test every 5 seconds. The VO2max criteria were defined as follow: (i) to attain a respiratory exchange ratio ≥ 1.1, (ii) to show a plateau in VO2 (change of <100 ml/min in the last 30 seconds stage), and (iii) to reach a maximal heart rate within 10 beats/min of the theoretical maximal heart rate (209-0.73 * age) [48]. Peak oxygen uptake value was considered when these criteria were not met [49].

The participants were instructed to fast for 3 hours before the VO2max test, and to avoid both caffeine consumption (i.e., 24 hours before) and PA (i.e., PA at moderate intensity 24 hours before and at vigorous intensity 48 hours before).

Muscular strength assessment

The determination of muscular strength parameters was performed on a different day (separated by 72-96 hours) but within the same week under similar previous conditions than the cardiorespiratory fitness assessment.

Lower limb muscular strength was determined through an isokinetic strength test (Gymnex Iso-2 dynamometer, Easytech s.r.l., Italy). We evaluated knee flexor and extensor concentric strength at $60^{\circ}\ s^{-1}$. We used safety belts to stabilize upper members, hip, and shoulders, aligning the lateral femoral condyle with the rotational axis of the dynamometer. Knee extension started at a joint angle of 90° and finished at 170°. A total of 5 submaximal attempts were established as a previous warm-up for each leg. After 1-minute resting, 3 maximal repetitions were performed following a previous validated protocol [50]. Extension and flexion peak torque was considered as the single repetition with the highest muscular force output (Nm). Strong verbal encouragement was given to the participants during the test.

A digital hand dynamometer (T.K.K. 5401 Grip-D; Takey, Tokyo, Japan) was used to determine hand grip strength (kg). The participants performed a total of two attempts with each hand resting 1-minute between them. They were asked to continuously squeeze for at least 2 seconds applying maximal intensity. Grip span was adjusted following the methodology described in a previous study [51]. We reported total hand grip strength as the sum of the best attempt of the right and left hands, respectively.

Both the VO₂max tests, the isokinetic strength tests, and the hand grip strength tests were conducted by the same research staff. VO₂max, extension and flexion peak torque, and hand grip strength were expressed in absolute values and in relation to weight and lean mass, respectively.

Dietary intake assessment

Dietary intake was obtained from three non-consecutive 24-hour dietary recalls collected on non-consecutive days, which included two weekdays and one weekend day. A trained and qualified research dietitian conducted a face-to-face interview, in which a meal sequence-based in detailed assessment and description of the food consumed by the participants were recorded. In order to assist participants in the estimation of the consumed food quantity, coloured photographs of different portions sizes of food were provided [52]. Dietary intake from the 24-hour recalls was analysed using EvalFINUT® software, which is based on USDA (US Department of Agriculture) and BEDCA ("Base de Datos Española de Composición de Alimentos") databases.

Blood samples collection

We collected blood samples (i.e., 10 ml) from the participant´s antecubital vein in the morning after 12 h fasting in ethylenediamine tetra-acetic acid-containing tubes and using the Vacutainer SST system (Becton Dickinson, Plymouth, UK). All samples were centrifuged at 4,000 rpm for seven minutes at 4ºC and stored at -80ºC. A DiaSorin Liaison® immunochemiluminometric analyzer (DiaSorin Ltd, Wokingham, Berkshire, UK) was used to assess plasma levels of 1,25(OH)₂D (pg/ml).

Statistical analysis

Visual check of histogram, Q-Q, box plots, and the Shapiro-Wilk test were used to check the normal distribution of all outcomes. The descriptive parameters are expressed as mean and standard deviation. Comparisons between men and women were performed with independent samples T-test. No interaction by sex was observed ($P>0.05$), hence the appropriateness of fitting models for men and women were combined including sex as a covariable.

Simple linear regression models were performed to examine the relationship of sedentary time, PA levels (i.e., LPA, MPA, VPA, MVPA), and physical fitness (i.e., VO₂max, extension and flexion peak torque, and hand grip strength) with 1,25(OH)₂D. Subsequently, multiple linear regressions analyses were performed to examine these relationships after adjusting for potential confounders. Model 1 was adjusted for age, Model 2 was adjusted for sex, Model 3 was adjusted

for fat mass percentage and Model 4 was adjusted for total energy, vitamin D, calcium, and phosphorus intake.

The level of significance was set at P<0.05. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, v. 25.0, IBM SPSS Statistics, IBM Corporation). Graphical plots were built using the GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

RESULTS

Descriptive characteristics of the participants by sex can be seen in Table 1. No significant differences were observed in 1,25(OH)$_2$D between men and women (P=0.6).

Figure 1 shows the association between sedentary time and PA levels with 1,25(OH)$_2$D in middle-aged sedentary adults. No association was observed between sedentary time, LPA, MPA, VPA and/or MVPA and 1,25(OH)$_2$D (all P≥0.28; Figure 1), which persisted after including age, sex, fat mass percentage and total energy, vitamin D, calcium and phosphorus intake in the model (all P≥0.21; Table 2).

Figure 2 shows the relationship between VO$_2$max and 1,25(OH)$_2$D in middle-aged sedentary adults. No significant association were observed between VO$_2$max expressed in absolute terms and relative to body weight and lean mass, and 1,25(OH)$_2$D (all P>0.09; Figure 2), which persisted after including age, sex, fat mass percentage and total energy, vitamin D, calcium and phosphorus intake in the model (all P≥0.07; Table 2).

We did not find any significant association of extension and flexion peak torque expressed in absolute terms and relative to body weight and lean mass with 1,25(OH)$_2$D (all P>0.2; Figure 3), which persisted after including age, sex, fat mass percentage and total energy, vitamin D, calcium and phosphorus intake in the model (all P≥0.17; Table 2).

Figure 4 shows the relationship between hand grip strength and 1,25(OH)$_2$D in middle-aged sedentary adults. No significant association were observed between hand grip, expressed in absolute terms and relative to body weight and lean mass, and 1,25(OH)$_2$D (all P>0.06; Figure 4).

All of these findings persisted once age, fat mass percentage and/or total energy, vitamin D, calcium and phosphorus intake were included in the statistical models (all P>0.1; Table 2).

Table 1. Descriptive characteristic of participants.

	N	All		N	Men		N	Women	
Age (years)	73	53.7	(5.1)	34	54.6	(5.2)	39	53.0	(5.0)
Anthropometric and body composition parameters									
Weight (kg)	73	75.7	(15.0)	34	87.4	(10.9)	39	65.3	(9.3)*
Height (cm)	73	167.7	(9.8)	34	175.7	(6.5)	39	160.7	(6.1) *
Body mass index (kg/m²)	73	26.7	(3.8)	34	28.3	(3.6)	39	25.3	(3.3)*
Fat mass (%)	73	40.1	(8.9)	34	34.7	(8.0)	39	44.5	(7.4)*
Fat mass (kg)	73	30.1	(8.5)	34	30.9	(9.8)	39	29.2	(7.1)
Lean mass (kg)	73	43.2	(11.7)	34	53.9	(6.5)	39	34.1	(5.8)*
Sedentary behaviour and physical activity levels									
Sedentary time (min/day)	70	745.9	(84.8)	33	770.7	(81.4)	37	723.7	(82.6)*
LPA (min/day)	70	173.7	(45.4)	33	169.0	(50.3)	37	177.8	(40.9)
MPA (min/day)	70	94.1	(35.0)	33	93.8	(35.3)	37	94.4	(35.3)
VPA (min/day)	70	1.7	(2.2)	33	2.3	(3.0)	37	1.1	(1.0)*
MVPA (min/day)	70	95.8	(35.6)	33	96.2	(35.9)	37	95.5	(35.8)
Physical fitness parameters									
VO$_2$max (ml/min)	70	2320.8	(643.4)	33	2893.9	(357.1)	37	1809.7	(332.5)*
VO$_2$max/weight (ml/kg/min)	70	30.3	(5.5)	33	33.1	(4.4)	37	27.9	(5.3)*
VO$_2$max/lean mass (ml/kg/min)	70	53.5	(7.9)	33	54.0	(7.0)	37	53.1	(8.8)
Extension peak torque (Nm)	72	264.0	(86.0)	34	332.4	(73.3)	38	202.8	(35.4)*
Extension peak torque/weight (Nm/kg)	72	3.5	(0.8)	34	3.8	(0.8)	38	3.1	(0.6)*
Extension peak torque/lean mass (Nm/kg)	72	6.1	(1.2)	34	6.2	(1.3)	38	6.0	(1.0)
Flexion peak torque (Nm)	72	123.8	(45.9)	34	157.4	(44.8)	38	93.6	(16.6)*
Flexion peak torque/weight (Nm/kg)	72	1.6	(0.5)	34	1.8	(0.6)	38	1.5	(0.3)*
Flexion peak torque/lean mass (Nm/kg)	72	2.9	(0.7)	34	2.9	(0.8)	38	2.8	(0.6)
Total hand grip (kg)	72	70.5	(23.5)	34	92.8	(12.2)	38	50.6	(8.2)*
Total hand grip/weight	72	0.9	(0.2)	34	1.1	(0.2)	38	0.8	(0.1)*
Total hand grip/lean mass	72	1.6	(0.2)	34	1.7	(0.2)	38	1.5	(0.2)*
Dietary Intake parameters									
Total Energy intake (kcal/day)	72	2071.7	(455.4)	34	2312.1	(402.9)	38	1854.6	(390.3)*
Vitamin D intake (µg/day)	72	5.0	(6.0)	34	3.8	(3.3)	38	6.1	(7.6)
Calcium intake (mg/day)	72	763.4	(340.5)	34	867.3	(396.9)	38	670.5	(251.4)*
Phosphorus intake (mg/day)	72	1324.7	(558.9)	34	1507.6	(689.6)	38	1161.0	(342.2)*
Blood parameters									
1,25 (OH)$_2$D (pg/ml)	73	40.3	(14.1)	34	38.3	(13.4)	39	42.0	(14.6)

Data are presented as means (standard deviation). *Significant differences between sexes obtained by the independent sample T test (P<0.05). LPA: Light physical activity time; MPA: Moderate physical activity time; VPA: Vigorous physical activity time; MVPA: Moderate-vigorous physical activity time; VO$_2$max: Maximal oxygen uptake; 1,25(OH)$_2$D: 1,25-dihydroxyvitamin D.

RESULTS AND DISCUSSION
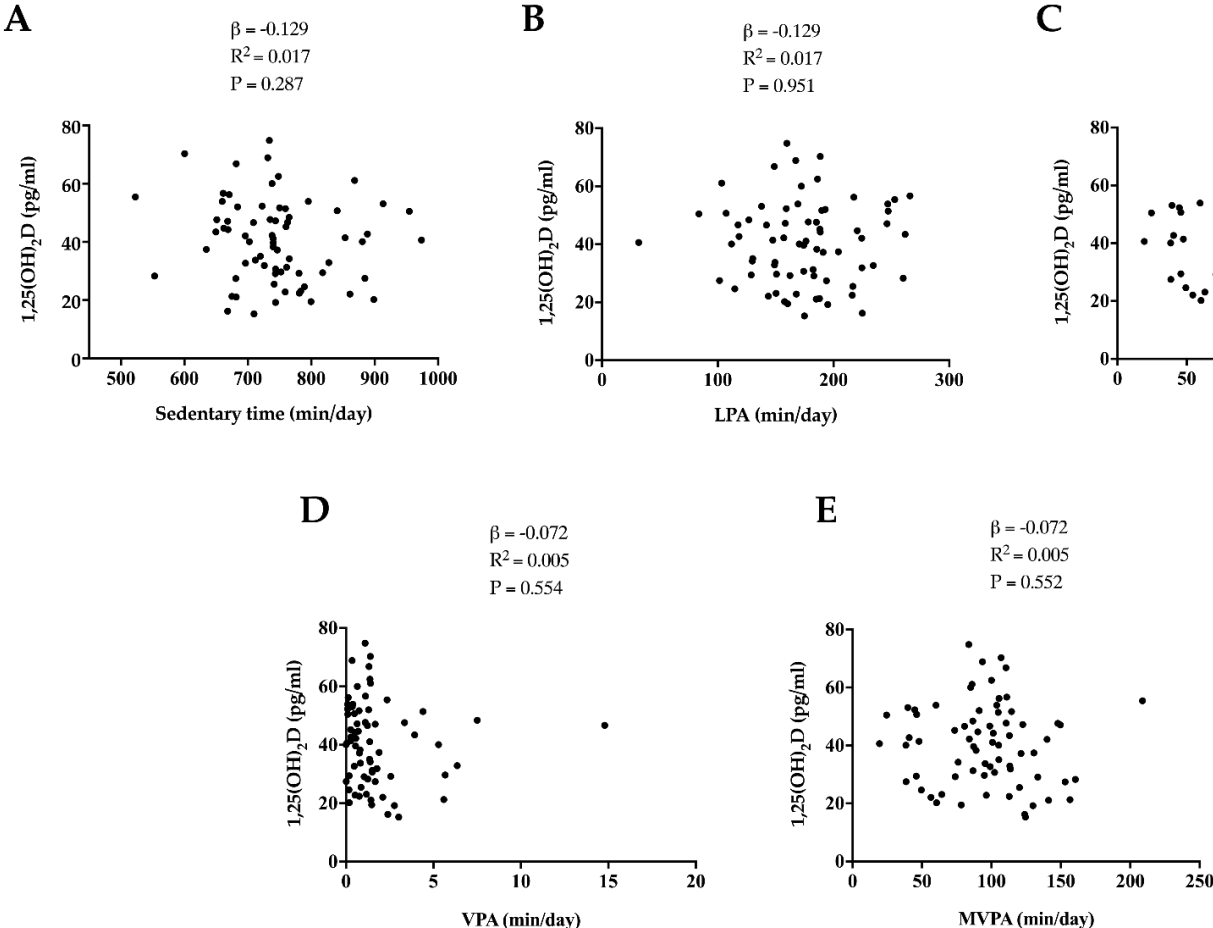
Figure 1. Association of sedentary time (Figure 1A), and physical activity intensity levels (Figure 1B, 1C, 1D and 1E) with 1,25 (standardized regression coefficient), R^2 and P value from a simple linear regression analysis. LPA: Light physical activity time; Vigorous physical activity time; MVPA: Moderate-vigorous physical activity time; 1,25(OH)$_2$D: 1,25-dihydroxyvitamin D.
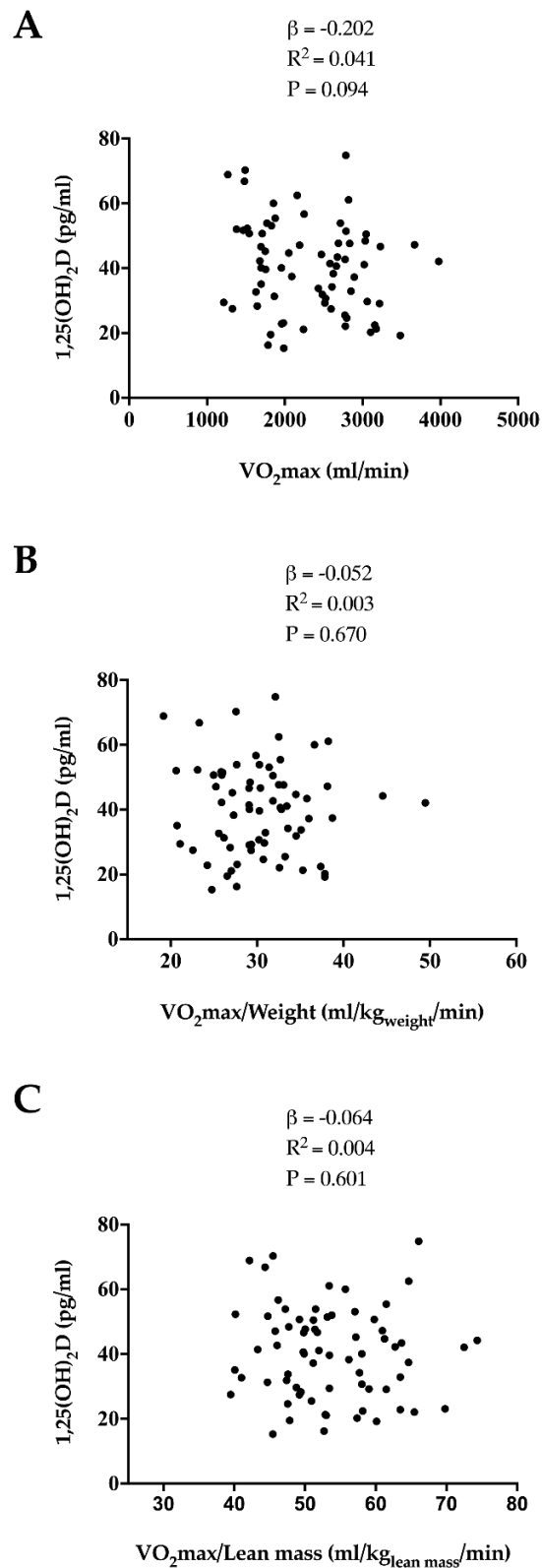
Figure 2. Association of maximal oxygen uptake (VO₂max) in absolute terms (Figure 2A), and relative to weight (Figure 2B) and lean mass (Figure 2C) with 1,25 (OH)₂D in middle-aged sedentary adults. β (standardized regression coefficient), R² and P value from a simple linear regression analysis. VO₂max: Maximal oxygen uptake; 1,25(OH)₂D: 1,25-dihydroxyvitamin D.

RESULTS AND DISCUSSION

A

β = -0.092
R² = 0.008
P = 0.443

B

β = -0.089
R² = 0.008
P = 0.459

C

β = 0.037
R² = 0.001
P = 0.757

D

β = 0.016
R² = 0.001
P = 0.893

E

β = 0.099
R² = 0.010
P = 0.410

F

β = 0.062
R² = 0.004
P = 0.607

Figure 3. Association of extension and flexion peak torque in absolute terms (Figure 3A and 3B), and relative to weight (Figure 3C and 3D) and lean mass (Figure 3E and 3F) with 1,25(OH)₂D in middle-aged sedentary adults. β (standardized regression coefficient), R² and P value from a simple linear regression analysis. 1,25(OH)₂D: 1,25-dihydroxyvitamin D.

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A

$\beta = -0.226$
$R^2 = 0.051$
$P = 0.056$

B

$\beta = -0.165$
$R^2 = 0.027$
$P = 0.166$

C

$\beta = -0.173$
$R^2 = 0.030$
$P = 0.146$

Figure 4. Association of hand grip strength in absolute terms (Figure 4A), and relative to weight (Figure 4B) and lean mass (Figure 4C) with 1,25(OH)$_2$D in middle-aged sedentary adults. β (standardized regression coefficient), R^2 and P value from a simple linear regression analysis. 1,25(OH)$_2$D: 1,25-dihydroxyvitamin D.

RESULTS AND DISCUSSION

Table 2. Association of sedentary time, physical activity intensity levels, VO₂max, extension and flexion peak torque, and total hand grip with 1,25(OH)₂D, adjusted for age (Model 1), sex (Model 2), fat mass percentage (Model 3) and total energy, vitamin D, calcium and phosphorus intake (Model 4).
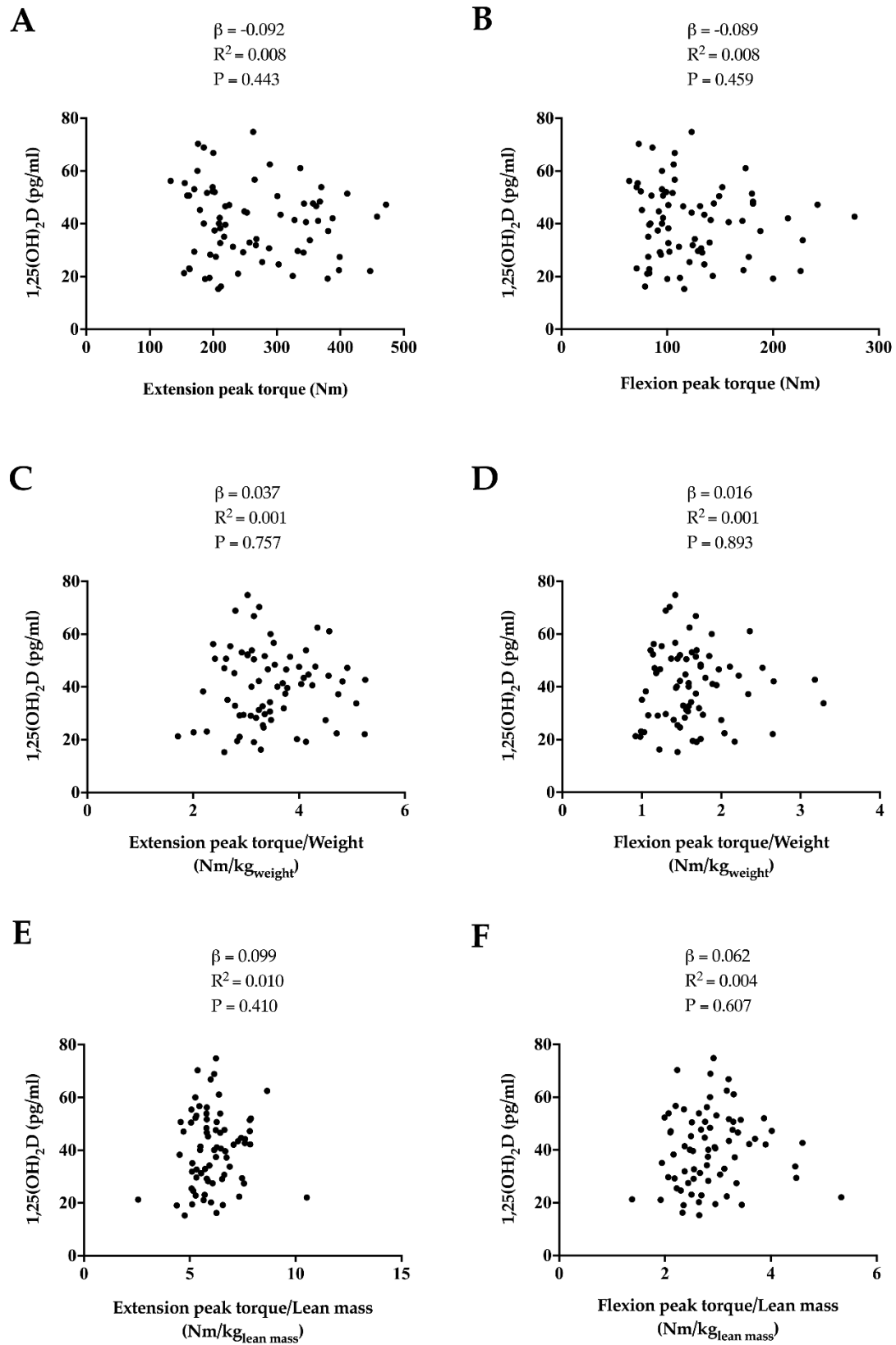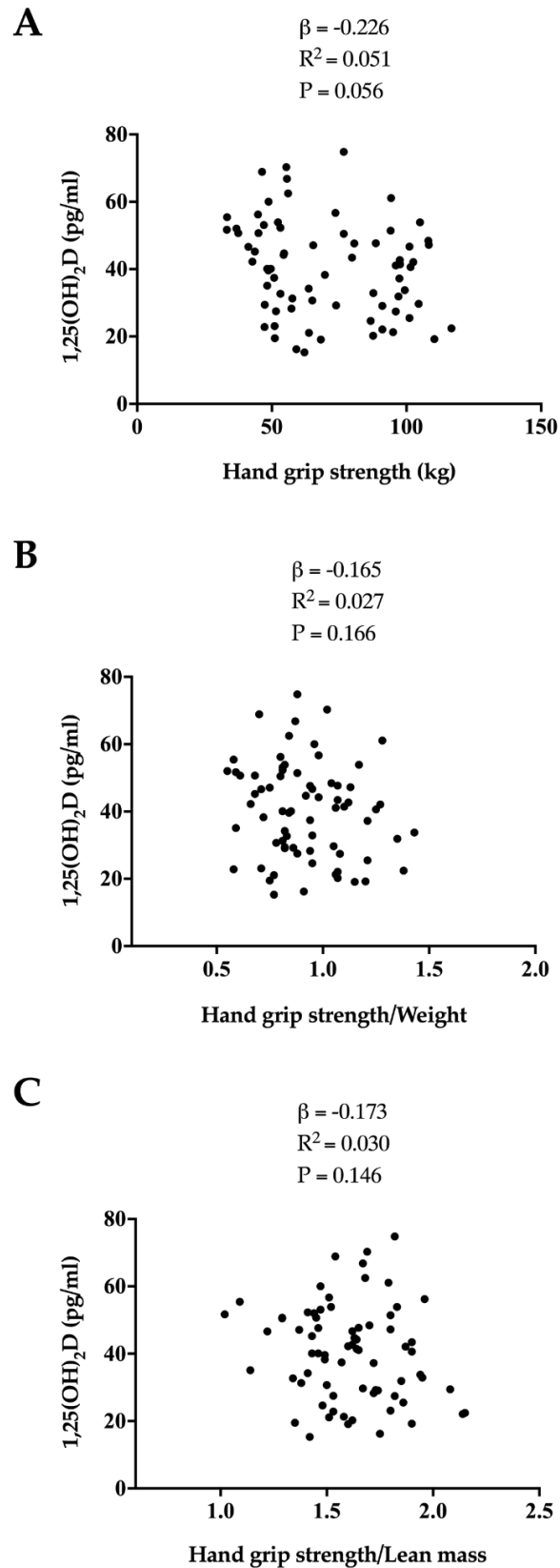
	1,25-dihydroxyvitamin D		
	β	R^2	P-value
Physical activity parameters			
Sedentary time (min/day)			
Model 1	-0.153	0.059	0.205
Model 2	-0.108	0.022	0.394
Model 3	-0.127	0.017	0.302
Model 4	-0.145	0.108	0.258
LPA (min/day)			
Model 1	0.001	0.036	0.993
Model 2	-0.003	0.011	0.982
Model 3	0.006	0.002	0.961
Model 4	-0.175	0.090	0.864
MPA (min/day)			
Model 1	-0.029	0.036	0.812
Model 2	-0.070	0.016	0.568
Model 3	-0.067	0.006	0.584
Model 4	-0.020	0.090	0.869
VPA (min/day)			
Model 1	-0.027	0.036	0.825
Model 2	-0.047	0.013	0.711
Model 3	-0.071	0.006	0.563
Model 4	0.013	0.090	0.922
MVPA (min/day)			
Model 1	-0.031	0.037	0.804
Model 2	-0.071	0.016	0.558
Model 3	-0.070	0.006	0.565
Model 4	-0.019	0.090	0.876
Cardiorespiratory fitness parameters			
VO₂max (ml/min)			
Model 1	-0.190	0.062	0.115
Model 2	-0.409	0.058	0.072
Model 3	-0.255	0.050	0.066
Model 4	-0.121	0.089	0.365
VO₂max/weight (ml/kg/min)			
Model 1	-0.033	0.027	0.784
Model 2	-0.005	0.010	0.971
Model 3	-0.070	0.003	0.656
Model 4	-0.051	0.080	0.681
VO₂max/lean mass (ml/kg/min)			
Model 1	-0.069	0.031	0.567
Model 2	-0.058	0.014	0.637
Model 3	-0.074	0.005	0.562
Model 4	-0.063	0.081	0.608

Table 2. Continued

	β	R²	P-value
Muscular strength parameters			
Extension peak torque (Nm)			
Model 1	-0.079	0.029	0.511
Model 2	0.024	0.018	0.895
Model 3	-0.071	0.010	0.604
Model 4	0.031	0.068	0.821
Extension peak torque/weight (Nm/kg)			
Model 1	0.050	0.025	0.679
Model 2	0.121	0.030	0.364
Model 3	0.119	0.016	0.413
Model 4	0.095	0.076	0.445
Extension peak torque/lean mass (Nm/kg)			
Model 1	0.096	0.032	0.420
Model 2	0.112	0.031	0.349
Model 3	0.088	0.014	0.470
Model 4	0.171	0.094	0.167
Flexion peak torque (Nm)			
Model 1	-0.084	0.030	0.482
Model 2	0.011	0.018	0.949
Model 3	-0.066	0.009	0.632
Model 4	-0.005	0.067	0.968
Flexion peak torque/weight (Nm/kg)			
Model 1	0.017	0.023	0.890
Model 2	0.082	0.024	0.529
Model 3	0.081	0.011	0.568
Model 4	0.036	0.068	0.771
Flexion peak torque/lean mass (Nm/kg)			
Model 1	0.049	0.025	0.684
Model 2	0.078	0.024	0.515
Model 3	0.058	0.009	0.630
Model 4	0.075	0.088	0.475
Total Hand grip (kg)			
Model 1	-0.211	0.066	0.076
Model 2	-0.588	0.077	**0.040**
Model 3	-0.275	0.056	0.061
Model 4	-0.159	0.086	0.251
Total Hand grip/weight			
Model 1	-0.153	0.046	0.200
Model 2	-0.140	0.028	0.409
Model 3	-0.258	0.033	0.169
Model 4	-0.135	0.084	0.282
Total Hand grip/lean mass			
Model 1	-0.179	0.054	0.131
Model 2	-0.141	0.033	0.311
Model 3	-0.168	0.034	0.160
Model 4	-0.127	0.082	0.318

Standardized β regression coefficient, R^2, and P-value of multiple-regression analysis are provided. LPA: Light physical activity time; MPA: Moderate physical activity time; VPA: Vigorous physical activity time; MVPA: Moderate-vigorous physical activity time; VO₂max: Maximal oxygen uptake; 1,25(OH)₂D: 1,25-dihydroxyvitamin D. Values in bold indicate significance differences ($P<0.05$).

DISCUSSION

This study sought to elucidate whether sedentary behavior, PA levels and physical fitness are related to 1,25(OH)$_2$D plasma levels in middle-aged adults. The present results show that objectively measured sedentary time and PA levels are not associated with 1,25(OH)$_2$D in middle-aged sedentary adults. Similarly, no association was found between physical fitness and 1,25(OH)$_2$D in middle-aged sedentary adults. These findings support the idea that although 1,25(OH)$_2$D could be related to the above-mentioned health markers -given their importance on physiological homeostasis-, it seems that 1,25(OH)$_2$D plasma levels are not related to either sedentary behaviour, PA levels and physical fitness in healthy individuals with relatively adequate values of 1,25(OH)$_2$D.

Sun exposure has been postulated as a key determinant of the associations between outdoor PA and circulating vitamin D concentration [53]. Moreover, previous studies [54] have suggested that PA can modulate specific vitamin D-related metabolites independently of sun exposure: (i) PA decreases plasma phosphate increasing by negative feedback 1,25(OH)$_2$D levels; and (ii) the reduction of ionized calcium during PA stimulate parathyroid hormone secretion subsequently increasing 1,25(OH)$_2$D [53]. Contrary to our initial hypothesis, no significant associations were observed between PA levels or sedentary behaviour and 1,25(OH)$_2$D, which did not concur with those obtained by Vanderschueren et al. [31] who found a positive association between PA levels and both 25(OH)D and 1,25(OH)$_2$D. These contradictory findings could be explained by different causes: (i) One of the inclusion criteria of our study was that the participants were not physically active (i.e., <20 min of PA on <3 days per week) and, therefore, we cannot know whether physically active individuals could present significantly higher 1,25(OH)$_2$D levels. (ii) We measured 1,25(OH)$_2$D plasma levels at the end of the summer in Granada (south of Spain) -which may overestimate the present values-, while the Vanderschueren et al. [31] assessments were conducted in different seasons of the year. (iii) Vanderschueren et al. [31] determined PA levels through subjective methods (i.e., questionnaires) whereas we objectively assessed PA by triaxial accelerometers.

Vitamin D deficiency is present in more than 50% of adults in developed countries [29] and has been related to an increased incidence of chronic cardiometabolic diseases and all-cause mortality [55]. Low levels of cardiorespiratory fitness is a well-recognized risk factor for cardiovascular morbidity and mortality [56]. There are several factors that influence VO$_2$max levels including cardiac output, arterial oxygen content, shunting of blood to myocytes, and extraction of oxygen by these myocytes [57]. Patients with myocardial hypertrophy, hypertension, and

endothelial dysfunction generally show low 25(OH)D plasma levels [29]. It is therefore plausible that a poor vitamin D status may decrease cardiac output and increase peripheral vessel resistance reducing VO$_2$max. [57]. In this sense, previous studies have showed a positive relationship between VO$_2$max and 25(OH)D in adults free of chronic diseases independently of confounders factors such as age, gender, BMI, and PA levels [57]. These results differ from those obtained in the present study since no significant associations were obtained between cardiorespiratory fitness and vitamin D status in our participant' cohort. The above-mentioned contradictory relationships could be due to the different vitamin D metabolite assessed (i.e., 25(OH)D vs. 1,25(OH)$_2$D) among other factors such as the different participant' ages, seasons in which measurements were made, etc. Hence, the current results support the idea that although 25(OH)D has been proposed as a key factor affecting cardiorespiratory fitness [57], it seems that 1,25(OH)$_2$D plasma levels are not related to VO$_2$max in healthy individuals with adequate values of these physiological parameters.

It has been previously reported that muscle strength is lowered with aging and that this drop begins during the fifth decade of life [58]. Interestingly, a significant reduction of muscular strength has been reported in individuals with a poor status of vitamin D [33–38]. Aspell et al. [59] showed that muscle strength (assessed by hand grip test) was negatively associated with 25(OH)D levels in a large sample (n=4157) of community-dwelling adults aged 60 years and over. Similar findings were obtained by Wang et al. [58] who observed a direct relationship of serum 25(OH)D concentration and hand grip strength in males aged above 50 years, independently of several confounding factors. These studies suggest that the effect of 25(OH)D on muscular strength might be mediated by 1,25(OH)$_2$D since this active metabolite regulates the synthesis of proteins that influence calcium transport and muscle contractility [60]. However, they did not assess 1,25(OH)$_2$D concentration claiming that this active metabolite should be determined in further studies. Our study shows, for the first time, that extension/flexion peak torque and hand grip strength are not associated with 1,25(OH)$_2$D concluding that 1,25(OH)$_2$D does not mediate the previously described relationship between 25(OH)D and muscular strength, at least, in middle-aged adults (i.e., aged 45-65 years old). Further studies are needed to confirm whether these findings apply to younger or older individuals.

Limitations and Strengths

Our study' findings should, however, be taken with caution as some limitations arise: (i) the cross-sectional design of the study does not allow us to make inferences about causality; (ii) our participants were middle-aged sedentary adults (45-65 years of age), hence, we do not know

whether these results can be extended to younger, older and/or physically active populations; and (iii) this study, like most clinical studies, was based on a single assay of 1,25(OH)$_2$D. Despite the aforementioned limitations, some strengths need to be considered: (i) we objectively measured sedentary time and PA intensity levels with a triaxial accelerometer, which can be considered one of the most important strengths of this study, since prior studies only employed subjective methods (i.e., questionnaires); (ii) cardiorespiratory fitness and muscular strength were measured using a gold-standard technology (i.e., maximum treadmill test using indirect calorimetry, and isokinetic strength test, respectively); and (iii) we registered dietary intake to control potential confounders.

CONCLUSIONS

In summary, our results suggest that sedentary time, PA intensity levels, and physical fitness are not associated with 1,25(OH)$_2$D plasma levels in middle-aged sedentary adults. Future interventional studies are necessary to improve understanding of whether a structured exercise intervention designed to increase both physical activity and physical fitness levels induce changes in 1,25(OH)$_2$D.

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Chapter 5

1,25-dihydroxyvitamin D and cardiometabolic risk factors in healthy sedentary adults: the FIT-AGEING study **(Study 3)**

ABSTRACT

Background & aims: A growing body of scientific works investigating the physio-pathological mechanisms behind cardiovascular disease has suggested that vitamin D deficiency could play a key role on its development. However, it remains unclear whether its active form (1,25-dihydroxyvitamin D [$1,25(OH)_2D$]) is associated with cardiometabolic risk factors in healthy individuals. The aim of the present study was to investigate the relationships of $1,25(OH)_2D$ plasma levels with cardiometabolic risk factors in a sample of healthy sedentary adults.

Methods: A total of 73 adults (~53% women; 53.7 ± 5.1 years old) were included in the current cross-sectional study. A sex-specific cardiometabolic risk score (MetScore) was calculated for each subject based on clinical parameters (i.e., waist circumference, systolic and diastolic blood pressure, plasma glucose, high-density lipoprotein cholesterol, and triglycerides) according to the International Diabetes Federation´s clinical criteria. Plasma levels of $1,25(OH)_2D$ were measured using a DiaSorin Liaison® immunochemiluminometric analyzer.

Results: No significant association was detected between $1,25(OH)_2D$ and MetScore ($\beta=0.037$, $R^2=0.001$, $P=0.770$), independently of age, sex and fat body mass index. A significant inverse association were observed between $1,25(OH)_2D$ and waist circumference ($\beta=-0,303$, $R^2=0.092$, $P=0.009$). These results were consistent after controlling by potential confounders.

Conclusions: The present results suggest that $1,25(OH)_2D$ plasma levels are associated with neither cardiometabolic risk factors nor insulin resistance in healthy sedentary adults. However, an inverse association of $1,25(OH)_2D$ plasma levels with central adiposity was observed in our study cohort

BACKGROUND

The incidence of chronic cardiometabolic disorders has dramatically increased during the last decades representing the leading cause of morbidity and mortality in the developed world [1–3]. Several cardiometabolic diseases (e.g., cardiovascular diseases or type II Diabetes Mellitus) are usually initiated by the presence of metabolic syndrome, which is defined as a clustering of abnormal physiological conditions (i.e., hypertension, central obesity, elevated triglycerides, glycaemic dysregulations, dyslipidaemia, and high concentrations of pro-inflammatory biomarkers) [4–7]. In this context, the identification of potential biomarkers capable of detect the risk and progression of cardiometabolic disease is a major goal of clinical medicine for promoting general health [8,9].

Vitamin D is a fat-soluble steroid pro-hormone endogenously synthesized as vitamin D_3 (cholecalciferol) in the skin upon exposure to ultraviolet B radiation from sunlight and/or obtained from the diet or vitamin D supplements as vitamin D_2 (ergocalciferol) or vitamin D_3 [10]. These pro-hormones are transported to the liver and subsequently hydroxylated producing the biologically inactive 25-hydroxyvitamin D [25(OH)D] [11,12]. 25(OH)D requires to be converted in 1,25-dihydroxyvitamin D (1,25(OH)$_2$D) by the 1-α-hydroxylase in the kidney to be biologically active [11,12]. 1,25(OH)$_2$D, also known as calcitriol, is, therefore, the main responsible of vitamin D biological functions [11,12].

Vitamin D deficiency is highly prevalent in different populations across the world [13–15]. This problem is mainly due to a decreased capacity to synthesize vitamin D from sunlight as well as increased body adiposity or low physical activity levels [13,16,17]. Vitamin D status has been also linked with a range of extra-skeletal properties (e.g., muscle function, cardiovascular homeostasis, nervous function, and immune response) beyond its key role on calcium/phosphate homeostasis [18,19]. In this sense, a growing body of scientific works investigating the physio-pathological mechanisms behind cardiometabolic disorders has suggested that vitamin D deficiency could play a key role in its development [20–23].

Previous studies have examined whether vitamin D deficiency -routinely measured as 25(OH)D– is associated with a higher risk of suffering cardiometabolic disease obtaining controversial findings [21,24–28]. However, considerably less attention has been paid to the relationship between the biologically active form of vitamin D (i.e., 1,25(OH)$_2$D) and cardiometabolic risk factors. Concretely, low 1,25(OH)$_2$D levels have been linked with glycaemic and lipid alterations in patients with psoriasis [29], and acute coronary syndrome [30]. It remains unclear whether 1,25(OH)$_2$D levels are associated with cardiometabolic risk factors in healthy

individuals. Given that identifying new potential biomarkers to detect cardiometabolic alterations in still healthy subjects potentially allows to apply preventive strategies and that they are preferable to the treatment of cardiometabolic diseases already established, it seems of scientific interest to determine whether $1,25(OH)_2D$ levels are associated with cardiometabolic risk factors in individuals free of chronic diseases [31,32]. Therefore, the present study aimed to investigate the relationships of $1,25(OH)_2D$ plasma levels with cardiometabolic risk factors in a sample of healthy sedentary adults.

MATERIALS AND METHODS

Study design and participants

The present study analyzed data from a sample of healthy sedentary adults (n=73 [~50% women]). The subjects included in this cross-sectional study were recruited from the FIT-AGEING study, a randomized controlled trial (clinicaltrial.gov: ID: NCT03334357), via social networks, electronic media, and leaflets. Data from the baseline assessment were collected during September-October 2016/17 at the *Centro de Investigación Deporte y Salud* (*CIDS*, Granada, Spain) and at the "Campus de la Salud" Hospital (Granada, Spain) and, subsequently used for the current study. Details concerning to the study design, procedures, and inclusion/exclusion criteria have been described in detail elsewhere [33]. Briefly, the inclusion criteria were: (i) to be aged between 45-65 years old, (ii) to be physically inactive (i.e., <20 min on 3 days/week), (iii) to present a stable body weight (i.e., body weight changes < 3kg) during the previous 3 months, (iv) to be a non-smoker, (v) to be non-pregnant, (vi) to be taking no long-term medication, and (vi) to have no cardiometabolic diseases. The FIT-AGEING study was approved by the Ethics Committee on Human Research at the University of Granada and the Regional Government of Andalucía [0838-N-2017]. The study protocols and experimental design were applied following the last revised ethical guidelines of the Declaration of Helsinki (last revision guidelines, 2013), with all participants providing written informed consent.

Anthropometric parameters and body composition

Body weight and height were measured using a Seca model 799 scale and stadiometer (Seca, Hamburg, Germany) to the nearest 0.1 kg and 0.1 cm respectively, with participants wearing lightweight clothes and barefoot. Body mass index (BMI) was then calculated as weight (kg)/height (m)^2. Waist circumference was registered according to the standard procedures of the

International Society for the Advancement of Kinanthropometry (ISAK) [34], and assessed in a standing position from the mid-point between the bottom of the rib cage and the iliac crest at the end of a normal expiration. Body composition analysis was performed using a dual-energy X-ray absorptiometer scanner (Discovery Wi, Hologic, Inc., Bedford, MA, USA), obtaining lean and fat body mass in kg following the manufacture's recommendations. From these measurements, fat body mass index (FMI) and lean body mass index (LMI) were calculated by the following equations:

$$FMI = \text{fat body mass [kg]/ height}^2 \text{[m])}$$

$$LMI = \text{lean body mass [kg]/ height}^2 \text{[m])}$$

Blood pressure

Systolic and diastolic blood pressure were measured with an Omrom® HEM 705 CP device (Omrom Health-care Co, Kyoto, Japan), an automated oscillometric sphygmomanometer that uses an upper arm cuff. The measurements were taken from the right arm with participants sitting and rested, following the most updated recommendations of the European Heart Society [35]. Readings were taken twice and the mean was subsequently calculated and used for further analysis. Mean blood pressure was calculated using the following formula: (systolic blood pressure – (diastolic blood pressure/3)) [35].

Blood samples

The blood samples were taken from the antecubital vein in the morning (8:30 AM – 10 AM) after overnight fasting and collected using the Vacutainer SST system (Becton Dickinson, Plymouth, UK) in ethylenediamine tetra-acetic acid-containing tubes. All samples were centrifuged at 4000 rpm for 7 min at 4 °C, aliquoted, and stored at –80 °C until further analyses. $1,25(OH)_2D$ plasma levels were measured using a DiaSorin Liaison® immunochemiluminometric analyzer (DiaSorin Ltd, Wokingham, Berkshire, UK) and expressed in pg/mL. Plasma glucose, triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL-C), alanine transaminase (ALT), and γ-glutamyl transferase (γ-GT) were determined using an AU5800 absorption spectrophotometer (Beckman Coulter, Brea, CA, USA). Plasma insulin was assessed by chemiluminescence immunoassay using a UniCel DxI 800 paramagnetic particles (Beckman Coulter, Brea, CA, USA). Low-density lipoprotein cholesterol (LDL-C) was calculated according to the following equation: LDL-C = (total cholesterol) – (HDL-C) – 0.45 x (triglycerides). Additionally, insulin/glucose, LDL-C/HDL-C, and triglycerides/HDL-C ratios were also calculated.

All blood samples were measured in the same laboratory located within the "Campus de la Salud" Hospital (Granada, Spain). All participants were requested to abstain from drugs and/or caffeine 24 hours before blood extraction, to refrain from any physical activity at moderate intensity (24 hours before) and/or vigorous intensity (48 hours before), and to eat a standardized dinner (i.e., egg omelette, boiled rice, and tomato sauce).

Cardiometabolic risk score

A sex-specific cardiometabolic risk score (MetScore) was calculated for each participant based on the clinical guidelines proposed by the International Diabetes Federation according to the following factors: waist circumference, mean blood pressure, plasma glucose, HDL-C, and triglycerides [36]. Standardized values were calculated for each variable as follows: Standardized values = (value – mean/standard deviation). The standardized HDL-C values were multiplied by -1 to indicate greater risk with higher values. MetScore was determined as the sum of these 5 standardized values divided by 5, to account for the number of variables included. This approach results in a continuous MetScore with a mean of 0 and a standard deviation of 1 by definition, considering lower values as a representation of a better cardiometabolic risk profile.

Quantitative insulin sensitivity check index (QUICKI) [37] was calculated from plasma insulin and glucose levels as:

$$QUICKI = 1/[\log(\text{plasma insulin (UI/mL)}) + \log(\text{plasma glucose (mg/dL)})]$$

The homeostasis model assessment for insulin resistance index (HOMA-IR) [38] was calculated as:

$$HOMA\text{-}IR = \text{plasma insulin (UI/mL)} \times \text{plasma glucose (nmol/L)}/22.5$$

Dietary intake

Dietary intake was collected via three 24-hour recalls on non-consecutive days (i.e., 2 days during the week and 1 day on the weekend) by qualified and trained dietitians through face-to-face interviews. The interviews were meal sequence-based where the subjects were asked to describe the different portion sizes of each food item they consumed using a colored photograph guide [39]. Energy, macronutrient, and micronutrient intake derived from food consumption were calculated using the EvalFINUT® software (FINUT, Granada, Spain), which is based on the USDA (United States Department of Agriculture) and BEDCA ("Base de Datos Española de Composición de Alimentos") databases.

Sedentary behaviour and physical activity

Objectively measured sedentary behavior and physical activity were assessed with a wrist-worn accelerometer (ActiGraph GT3X+, Pensacola, FL, United States) for seven consecutive days (24 hours/day) [33]. Participants were requested to wear the accelerometers constantly, except during bathing or aquatic activities such as swimming. The ActiGraph sampling frequency was initialized to store raw acceleration information at a rate of 100 Hz [40]. The accelerometry data collection were exported and processed using the ActiLife v.6.13.3 software (ActiGraph, Pensacola, FL, United States) and the GGIR package (v.1.5-12, https://cran.r-project.org/web/packages/GGIR/) in R software (v.3.1.2, https://www.cran.r-project.org/) [41,42]. Time spent at various levels of movement intensity (i.e., moderate-vigorous) was determined according to age-specific cut-points for Euclidean Norm Minus One [41]. Data from participants with at least 16 hours of daily accelerometer wear time for 4 days (including 1 weekend day) were included in the analyses.

Statistical analyses

The Shapiro-Wilk test, visual check of histograms, Q-Q, and box plots were used to verify the distribution of all variables. The descriptive parameters are reported as mean and standard deviation. Sex differences for each variable were performed using an unpaired sample t-test. There were no significant sex interactions between 1,25(OH)$_2$D plasma levels and all cardiometabolic risk factors (all P>0.05). The analyses were thus performed including both men and women together.

We conducted simple linear regression models to examine the association between 1,25(OH)$_2$D plasma levels and MetScore, QUICKI, and HOMA-IR. Hierarchical regression analyses were subsequently performed in order to check whether 1,25(OH)$_2$D plasma levels predict the above-mentioned outcomes independently of potential confounders based on theoretical and statistical considerations. The entry order of potential confounder in the hierarchical analysis were as follows: age, sex, BMI, FMI, LMI, total energy intake, vitamin D intake, total physical activity and sedentary behavior. Multiple linear regression analyses were built using the derived confounders from the hierarchical regression analyses. Similar analyses were conducted to study the association between 1,25(OH)$_2$D plasma levels and the remaining cardiometabolic risk factors.

All analyses were performed using the Statistical Package for Social Sciences (SPSS, v. 22.0, IBM SPSS Statistics, IBM Corporation, Armonk, NY, USA). Graphical plots were generated using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Statistical significance was defined as P values <0.05 for all analyses.

RESULTS

The baseline characteristics of the participants are shown in Table 1. No significant differences were observed in 1,25(OH)$_2$D plasma levels between men and women (P=0.576).

Simple linear regression analysis revealed no associations of 1,25(OH)$_2$D plasma levels with MetScore (β=0.037, R^2=0.001, P=0.770; Figure. 1A), QUICKI (β=0.011, R^2=0.001, P=0.929; Figure 1B) and HOMA-IR (β=0.005, R^2=-0.015, P=0.965; Figure 1C).

Based on this hierarchical regression, we discarded LMI, total energy intake, vitamin D intake, total physical activity and sedentary behavior as confounders outcomes (all P>0.05 and all Sig. F change >0.05), only including age, sex, BMI, and FMI as potential confounders (Table 2). The results persisted when the analyzes were adjusted for age, sex, BMI, and/or FMI (all P$\geq$0.59; Table 3).

Table 4 shows the associations between 1,25(OH)$_2$D and cardiometabolic risk factors. A significant slightly negative association was observed between 1,25(OH)$_2$D and waist circumference (β=-0,303, R^2=0.092, P=0.009), which remained statistically significant after adjusting for age, sex, and FMI (Table 4; all P$\leq$0.038). There was a significant slightly positive association between 1,25(OH)$_2$D and diastolic blood pressure (P=0.033), which was partially attenuated after adjusting for potential confounders (all P$\leq$0.086; Table 4). Similarly, we found a significant positive association between 1,25(OH)$_2$D and total cholesterol (β=0,267, R^2=0.071, P=0.025), which was attenuated once age, sex and FMI were included in the model (all P$\leq$0.111). No significant association was found between 1,25(OH)$_2$D and others cardiometabolic risk factors (Table 4; all P>0.05).

Table 1. Characteristics of participants at baseline.

	N	All		N	Men		N	Women	
Age (years)	73	53.7	(5.1)	34	54.6	(5.2)	39	53.0	(5.0)
1,25(OH)$_2$D (pg/ml)	73	40.3	(14.1)	34	38.3	(13.4)	39	42.0	(14.6)
Anthropometric and body composition									
Weight (kg)	73	75.5	(15.0)	34	87.3	(11.1)	39	65.3	(9.3)*
Body mass index (kg/m^2)	73	26.7	(3.8)	34	28.3	(3.7)	39	25.3	(3.3)*
Waist circumference (cm)	73	95.0	(11.8)	34	102.8	(8.9)	39	88.2	(9.7)*
Fat body mass (%)	73	40.1	(9.0)	34	35.0	(8.0)	39	44.5	(7.4)*
Fat body mass (kg)	73	30.1	(8.5)	34	31.0	(9.8)	39	29.2	(7.1)
Lean body mass (kg)	73	43.2	(11.5)	34	53.6	(6.4)	39	34.1	(5.8)*
Blood Pressure									
Systolic blood pressure (mm Hg)	66	126.9	(15.8)	30	134.0	(14.0)	36	120.9	(14.8)*
Diastolic blood pressure (mm Hg)	66	81.1	(11.8)	30	85.1	(11.1)	36	77.6	(11.4)*
Mean blood pressure (mm Hg)	66	104.0	(13.2)	30	109.6	(11.9)	36	99.3	(12.5)*
Glucose Metabolism									
Plasma glucose (mg/dL)	70	93.6	(11.4)	33	95.0	(13.6)	37	92.3	(8.9)
Plasma insulin (UI/mL)	70	8.1	(5.7)	33	8.9	(6.7)	37	7.3	(4.5)
Insulin glucose ratio	70	12.6	(7.6)	33	13.4	(8.1)	37	11.9	(7.1)
QUICKI	70	0.362	(0.036)	33	0.357	(0.039)	37	0.365	(0.033)
HOMA-IR	70	1.79	(1.19)	33	1.91	(1,26)	37	1.69	(1.12)
Lipid Metabolism									
Total cholesterol (mg/dL)	70	206.4	(31.9)	33	200.7	(32.3)	37	211.5	(31.0)
HDL-C (mg/dL)	70	134.2	(68.2)	33	55.3	(12.9)	37	61.7	(11.1)*
LDL-C (mg/dL)	70	58.7	(12.3)	33	125.1	(27.9)	37	127.3	(26.6)
Triglycerides (mg/dL)	70	126.2	(27.1)	33	144.8	(83.7)	37	124.8	(49.9)
LDL-C/HDL-C	70	2.31	(0.90)	33	2.45	(0.96)	37	2.18	(0.84)
Triglycerides/ HDL-C	70	2.57	(1.92)	33	3.02	(2.39)	37	2.16	(1.25)
MetScore	66	-0.0002	(0.3414)	30	0.0187	(0.3836)	36	-0.0160	(0.3065)
Dietary Intake									
Total Energy (kcal/day)	72	2094.9	(478.8)	34	2302.8	(466.6)	38	1909.0	(413.0)*
Fat (g/day)	72	87.5	(25.0)	34	97.6	(24.4)	38	78.4	(22.2)*
Carbohydrate (g/day)	72	218.3	(70.2)	34	238.3	(75.0)	38	200.4	(61.1)*
Protein (g/day)	72	89.0	(34.5)	34	92.4	(30.8)	38	86.1	(37.6)
Ethanol (g/day)	72	11.2	(13.2)	34	16.2	(16.3)	38	6.6	(7.2)
Vitamin D (μg/day)	72	5.0	(6.0)	34	3.8	(3.3)	38	6.1	(7.6)
Calcium (mg/day)	72	763.4	(340.5)	34	867.3	(396.9)	38	670.5	(251.4)*
Phosphorus (mg/day)	72	1324.7	(558.9)	34	1507.6	(689.6)	38	1161.0	(342.2)*
Physical activity levels									
Sedentary time (min/day)	70	745.9	(84.8)	33	770.7	(81.4)	37	723.7	(82.6)*
Total physical activity (min/day)	70	269.5	(75.1)	33	265.2	(79.3)	37	273.3	(72.0)

Data are shown as means (standard deviation). *Significant differences between sexes obtained by the independent sample T test (P<0.05). 1,25(OH)$_2$D: 1,25-dihydroxyvitamin D; QUICKI: Quantitative insulin sensitivity check index; HOMA-IR: Homeostasis model assessment for insulin resistance index; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; MetScore: Cardiometabolic risk score.

RESULTS AND DISCUSSION

A

$\beta = 0.037$
$R^2 = 0.001$
$P = 0.770$

B

$\beta = 0.011$
$R^2 = 0.001$
$P = 0.929$

C

$\beta = 0.005$
$R^2 = 0.001$
$P = 0.965$
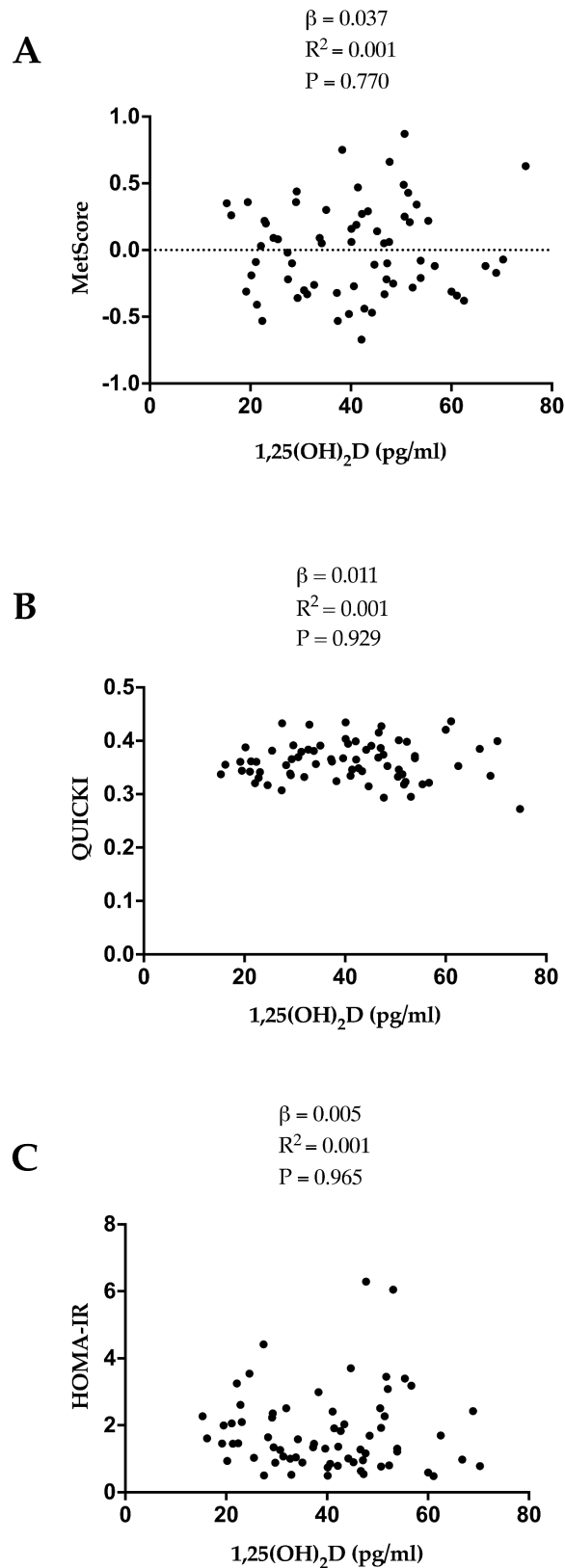

Figure 1. Association between 1,25(OH)2D and the cardiometabolic risk score (MetScore, Figure 1A), the quantitative insulin sensitivity check index (QUICKI, Figure 1B), and the homeostasis model assessment of insulin resistance index (HOMA-IR, Figure 1C) in healthy sedentary adults. β: Standardized regression coefficient; R² and P value are provided for simple linear regression analysis.

Table 2. Hierarchical regression between 1,25(OH)$_2$D levels with MetScore, QUICKI, and HOMA-IR

	MetScore				QUICKI			
	β	R^2 change	Sig. F change	P-value	β	R^2 change	Sig. F change	P-value
1,25(OH)$_2$D (pg/ml)								
Age (years)	0.183	0.151	**0.002**	0.168	-0.315	0.112	**0.006**	**0.024**
Sex	-0.447	0.001	0.784	**0.036**	0.252	0.001	0.871	0.243
Body mass index (kg/m^2)	-2.302	0.037	0.108	0.100	0.269	0.055	**0.045**	0.855
Fat body mass index (kg/m^2)	2.295	0.144	**0.001**	**0.045**	-0.417	0.017	0.254	0.733
Lean body mass index (kg/m^2)	1.404	0.027	0.129	0.184	-0.017	0.001	0.751	0.988
Total energy intake (kcal/day)	-0.200	0.017	0.227	0.122	0.160	0.037	0.095	0.220
Vitamin D intake (μg/day)	0.165	0.016	0.239	0.232	0.228	0.037	0.092	0.087
Total physical activity time (min/day)	-0.064	0.004	0.557	0.557	-0.180	0.031	0.115	0.115
Total sedentary time (min/day)	0.017	0.004	0.841	0.948	-0.052	0.032	0.286	0.845

β: Standardized regression coefficient; R^2; and P value were obtained from the hierarchical multiple linear regression analyses. 1,2! Cardiometabolic risk score; QUICKI: Quantitative insulin sensitivity check index; HOMA-IR: Homeostasis model assessment for insu

Table 3. Association of 1,25(OH)₂D levels with MetScore, QUICKI and HOMA.

	All (N = 73)		
	β	R^2	P-value
Cardiometabolic risk score			
Model 1	-0.023	0.150	0.848
Model 2	-0.025	0.150	0.832
Model 3	0.051	0.274	0.654
QUICKI			
Model 1	0.063	0.103	0.591
Model 4	-0.004	0.152	0.976
HOMA-IR			
Model 1	-0.023	0.150	0.848

Linear regression analyses were performed, adjusting for age (Model 1), age and sex (Model 2), age, sex and FMI (Model 3), age and BMI (Model 4). Standardized β regression coefficient, adjusted R^2, and P value of multiple-regression analysis are provided. Abbreviations: 1,25(OH)₂D, 1,25-dihydroxyvitamin D; QUICKI, quantitative insulin sensitivity check index; HOMA-IR, homeostasis model assessment for insulin resistance index.

Table 4. Association between 1,25(OH)₂D levels and cardiometabolic risk factors.

	All (N = 73)		
	β	R^2	P-value
Weight (kg)			
Model 0	-0.217	0.047	0.065
Model 1	-0.054	0.050	0.080
Model 2	-0.088	0.593	0.287
Model 3	-0.030	0.800	0.593
Waist circumference (cm)			
Model 0	**-0.303**	**0.092**	**0.009**
Model 1	**-0.296**	**0.093**	**0.012**
Model 2	**-0.197**	**0.457**	**0.034**
Model 3	**-0.125**	**0.777**	**0.038**
Systolic blood pressure (mm Hg)			
Model 0	0.166	0.028	0.182
Model 1	0.076	0.369	0.454
Model 2	0.107	0.467	0.261
Model 3	0.100	0.468	0.305
Diastolic blood pressure (mm Hg)			
Model 0	**0.262**	**0.069**	**0.033**
Model 1	0.185	0.324	0.083
Model 2	**0.208**	**0.380**	**0.045**
Model 3	**0.212**	**0.380**	**0.048**
Mean blood pressure (mm Hg)			
Model 0	0.217	0.047	0.080
Model 1	0.128	0.378	0.207
Model 2	0.157	0.464	0.102
Model 3	0.155	0.464	0.117

Table 4. Continued

| | All (N = 73) | | |
	β	R^2	P-value
Glucose (mg/dL)			
Model 0	-0.008	<0.001	0.947
Model 1	-0.006	<0.001	0.958
Model 2	0.010	0.016	0.934
Model 3	0.036	0.036	0.775
Insulin (UI/mL)			
Model 0	0.185	0.034	0.126
Model 1	0.133	0.134	0.252
Model 2	0.147	0.145	0.210
Model 3	0.193	0.207	0.098
Insulin glucose ratio			
Model 0	0.147	0.022	0.225
Model 1	0.085	0.165	0.456
Model 2	0.091	0.167	0.429
Model 3	0.135	0.225	0.238
Total cholesterol (mg/dL)			
Model 0	**0.267**	**0.071**	**0.025**
Model 1	0.203	0.224	0.067
Model 2	0.173	0.273	0.111
Model 3	0.203	0.301	0.063
Triglycerides (mg/dL)			
Model 0	0.080	0.006	0.512
Model 1	0.017	0.155	0.884
Model 2	0.028	0.163	0.806
Model 3	0.052	0.179	0.658
HDL-C (mg/dL)			
Model 0	-0.182	0.033	0.132
Model 1	-0.103	0.265	0.336
Model 2	-0.129	0.303	0.223
Model 3	-0.138	0.305	0.204
LDL-C (mg/dL)			
Model 0	0.124	0..015	0.305
Model 1	0.038	0.296	0.716
Model 2	0.020	0.313	0.846
Model 3	0.034	0.319	0.743
LDL-C/HDL-C			
Model 0	0.172	0.030	0.154
Model 1	0.081	0.342	0.425
Model 2	0.089	0.346	0.383
Model 3	0.110	0.359	0.292
Tryglycerides/HDL-C			
Model 0	0.102	0.010	0.403
Model 1	0.030	0.200	0.784
Model 2	0.052	0.226	0.638
Model 3	0.073	0.239	0.517

Linear regression analyses were performed, unadjusted (Model 0), adjusting for age (Model 1), age and sex (Model 2), age, sex and FMI (Model 3). Standardized β regression coefficient, adjusted R^2, and P value of multiple-regression analysis are provided. $1,25(OH)_2D$: 1,25-dihydroxyvitamin D; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol.

DISCUSSION

The current study sought to elucidate whether $1,25(OH)_2D$ plasma levels are related to cardiometabolic risk factors in sedentary adults free of chronic cardiometabolic diseases. Our results show that $1,25(OH)_2D$ plasma levels are associated with neither the MetScore nor insulin resistance in healthy sedentary adults. However, we observed that higher $1,25(OH)_2D$ plasma levels were consistently associated with low central adiposity in our study cohort. These findings support the idea that although $1,25(OH)_2D$ has been proposed as a key factor affecting cardiometabolic health in patients with chronic diseases [29,30], it seems that $1,25(OH)_2D$ plasma levels are not related to cardiometabolic risk factors in healthy individuals with adequate values of these physiological parameters.

$1,25(OH)_2D$ plays a crucial role in mineral homeostasis and skeletal health being its deficiency classically related to rickets in children and osteomalacia in adults [43]. Although its main function on the skeletal system is to modulate calcium and phosphorus metabolism through bone resorption, renal retention or intestinal absorption, vitamin D metabolites also exert important physiological functions in other tissues [44]. Indeed, previous studies have reported its implication on several chronic pathologies (e.g., skin and autoimmune disorders, cancer, type II diabetes mellitus, hypertension, or cardiovascular disease) [45].

Vitamin D deficiency is currently considered as a serious global problem [46] being the lower skin synthesis (as a consequence of the aging process) and others environmental (e.g., sunlight exposure, season or geographical localization) factors its main cause [43]. It has been reported that the prevalence of vitamin D deficiency depends on age, gender, geographical latitude or ethnicity [43]. Concretely, an increased incidence of vitamin D deficiency has been described in elderly individuals with cardiovascular diseases [47]. However, excessive levels of vitamin D have been also associated with cardiovascular disease-related problems including renal impairment and kidney stones, among others [47].

Several molecular and physiological pathways have been described as an explanation of the mechanistic basis of the influence of $1,25(OH)_2D$ on cardiovascular function [44]. Experimental studies have demonstrated the important role of $1,25(OH)_2D$ on the immune and inflammatory system during the pathogenesis of cardiovascular disorders such as atherosclerosis, aneurysm development, and other inflammatory vascular diseases [48]. Specifically, Beilfuss et al. showed that vitamin D supplementation produced a significant reduction of IL-6 plasma levels in overweight individuals [49]. Moreover, Amer and Qayyum reported a negative association between $25(OH)D$ circulating levels and C-reactive protein concentrations in apparently healthy adults

suggesting its important influence on T cell regulation [50]. On the other hand, Pilz et al. showed that 1,25(OH)$_2$D exerts a direct effect on lipid profile (i.e., via reducing triglyceride levels or ApoA1 expression) or indirectly by defeating lipolysis through decreasing parathyroid hormone release [51]. Furthermore, 1,25(OH)$_2$D also inhibits foam cell formation increasing cholesterol efflux [52].

Playford et al. demonstrated that circulating 1,25(OH)$_2$D levels were inversely associated with markers of visceral adiposity, vascular uptake of F-fluorodeoxyglucose (FDG), and coronary plaque burden independently of cardiometabolic risk factors in patients with psoriasis [29], which partially concur with our current findings. However, while we showed a positive association between 1,25(OH)$_2$D plasma levels and low central adiposity in sedentary but healthy individuals, no significant relationships were obtained between 1,25(OH)$_2$D plasma levels and neither the MetScore nor insulin resistance in our study cohort. The presently observed lack of associations might be explained by the fact that our study subjects were all healthy individuals with 1,25(OH)$_2$D and cardiometabolic risk-related factors within normal ranges. The 1,25(OH)$_2$D normal values obtained in our study cohort could be a consequence of their higher sun exposure - blood samples at the baseline were collected in September in the south of Spain- compared with those obtained by other people living in countries far from the equator [53].

Limitations

The present study suffers from several limitations. Firstly, the cross-sectional design precluded us from making causal conclusions about the association of 1,25(OH)$_2$D plasma levels with cardiometabolic risk factors. Secondly, based on the inclusion criteria of the present study, our findings only apply for healthy sedentary adults (45-65 years old); hence, they may not be generalizable to other populations such as older, younger, trained, and/or diseased individuals. Thirdly, we have no data on 25-hydroxyvitamin D plasma levels, which would be desirable to well-understand our study findings. Finally, since the relatively small sample size of the present study, the data should be interpreted with caution.

CONCLUSIONS

In summary, the present results suggest that 1,25(OH)$_2$D plasma levels are associated with neither cardiometabolic risk factors nor insulin resistance in healthy sedentary adults, independently of several confounders. However, an inverse association of 1,25(OH)$_2$D plasma levels with central adiposity was observed in our study cohort. These results have important clinical implications

since they suggest that $1,25(OH)_2D$ seems to be related to central adiposity in healthy individuals with normal values of these physiological parameters but do not to others key cardiometabolic risk factors. Our study therefore highlights the importance of including the measurement of $1,25(OH)_2D$ when investigating the effects of sunlight exposure of vitamin D supplementation on the prevention and/or treatment of cardiovascular disease.

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Chapter 6

1,25-dihydroxyvitamin D and S-Klotho plasma levels: the relationship between two renal anti-aging biomarkers mediated by bone mineral density in middle-aged sedentary adults **(Study 4)**

ABSTRACT

Background & aims: The main active metabolite of vitamin D, the 1,25-dihydroxyvitamin D (1,25(OH)$_2$D), and the shed form of the α-Klotho gene (S-Klotho) play an important role in aging-related physiological processes and are currently considered powerful anti-aging renal biomarkers. We aimed to investigate the relationship between 1,25(OH)$_2$D and S-Klotho plasma levels in middle-aged sedentary healthy adults. We also aimed to study the mediation role of body composition, physical activity levels, dietary parameters and blood markers in the association between 1,25(OH)$_2$D and S-Klotho plasma levels.

Methods: A total of 73 middle-aged sedentary adults (53.4% women; 53.7 ± 5.1 years old) were enrolled in this cross-sectional study. 1,25(OH)$_2$D plasma levels were measured using a DiaSorin Liaison® immunochemiluminometric analyzer. S-Klotho plasma levels were measured using a solid-phase sandwich enzyme-linked immunosorbent assay. Body composition analysis was performed using dual-energy-X-ray absorptiometry scanner (DXA).

Results: A tendency toward a negative association was observed between 1,25(OH)$_2$D and S-Klotho plasma levels (β=-0.222, R^2=0.049, P=0.059). The association was attenuated after controlling for age and sex and become significant after controlling for fat mass index. In addition, the association between 1,25(OH)$_2$D and S-Klotho levels was indirectly influenced by bone mineral density, with a percentage of mediation of 31.40%.

Conclusions: Our study shows that 1,25(OH)$_2$D is negatively associated with S-Klotho plasma levels in middle-aged sedentary adults, which is partially mediated by bone mineral density.

BACKGROUND

Aging is a natural and multi-factorial process characterized by a progressive decline of physiological integrity leading to impaired physical and cognitive functions, and increasing the incidence of several age-related diseases (i.e., cardiovascular disorders, osteoporosis, diabetes, cancer, and neurodegenerative diseases, among others) [1–4]. These diseases represent the major cause of morbidity and mortality in developed countries, causing important public health problems and economic burden [5,6]. The discovery of specific anti-aging biomarkers, as well as their physiological functions and interactions, have received considerable attention during the last decades aiming to detect therapeutic targets to promote personalized interventions to improve human' health and longevity [7].

Recent studies have established the importance of vitamin D status on human' health during the ageing process beyond its role on specific physiological mechanisms in different organs and system [8]. Vitamin D alterations have been consistently linked to greater incidence and prevalence of several age-related chronic diseases, impaired physical function, and mortality in different populations [9–11]. In the kidney, the 25-hidroxyvitamin D undergoes hydroxylation by 25-hydroxyvitamin D-1-α-hydroxylase to form 1,25(OH)$_2$D which is the main active metabolite and the responsible of the majority of functions [12]. Previous studies have reported important anti-aging functions of 1,25(OH)$_2$D specifically in bone mineral metabolism [13], oxidative stress [14], neurological functions [15], energy metabolism [16] and cardiovascular health [17].

In humans, the secreted form of the α-Klotho gene (S-Klotho) has been proposed as an accurate indicator of renal α-Klotho protein expression [18], an anti-aging gene which extends life expectancy when is over-expressed, and accelerates aging-like phenotypes when is under-expressed [19]. S-Klotho is therefore considered a powerful anti-aging biomarker in healthy humans [20] which could maintain a reciprocal interaction with 1,25(OH)$_2$D [21]. Murine studies [22–25] have shown that 1,25(OH)$_2$D stimulates the expression of the α-Klotho gene and fibroblast growth factor (FGF) 23 while increased levels of S-Klotho and FGF23 inhibit 1-α-hydroxylase leading to the lowering 1,25(OH)$_2$D synthesis and its subsequent degradation [21]. However, to the best of our knowledge, there is no study investigating this relationship in humans. Understanding the interaction between these two anti-aging biomarkers in middle-aged adults is of clinical interest since, it has been previously established that interventions to delay age-related diseases are preferable when individuals are still relatively young and healthy [2,7].

Therefore, the aim of the present study was to investigate the relationship between 1,25(OH)$_2$D and S-Klotho plasma levels in middle-aged sedentary adults. Given that 1,25(OH)$_2$D

and S-Klotho plasma levels have been associated with the body composition status in humans [26,27], we also investigated the mediation role of body composition in the association between 1,25(OH)$_2$D and S-Klotho plasma levels.

MATERIALS AND METHODS

Study design and participants

The participants included in this cross-sectional study were engaged as part of the FIT-AGEING project, an exercise-based randomised controlled trial (clinicaltrial.gov: ID: NCT03334357). The study sample was recruited in Granada (Spain) via electronic media, social networks, and leaflets. Details concerning the inclusion and exclusion criteria can be seen elsewhere [28]. Briefly, a total of seventy-three middle-aged sedentary healthy adults (39 women) aged between 45 and 65 years participated in the current study. They reported to be non-physically active (i.e., less than 20 min of physical activity on less than 3 days/week), to have stable weight (weight changes < 3 kg) in the past 12 weeks, to be free of disease, not to be pregnant, to be non-smoker, and not taking any medication. An extensive medical examination was performed before the beginning of the study. Design, rationale, and methodology of the study were approved by the Ethics Committee on Human Research of the Regional Government of Andalucía (CEI-Granada) (0838-N-2017) and all participants provided oral and written informed consent in accordance with the last revised ethical guidelines of the Declaration of Helsinki (2013). All baseline assessments were conducted between September and October of 2016/2017 at the *Centro de Investigación Deporte y Salud* (*CIDS*, Granada, Spain) and at the "Campus de la Salud" Hospital (Granada, Spain).

Blood samples assessment

Blood samples were obtained from the antecubital vein after overnight fasting and in resting conditions (at least 10 minutes before) in a supine position. All participants were requested (i) to abstain from drugs and/or caffeine, (ii) not to do any physical activity at moderate intensity (24 hours before) and/or vigorous intensity (48 hours before), and (iii) to eat an established dinner before sampling (i.e., boiled rice and egg omelette with fried tomato sauce). Blood samples were collected in prechilled Ethylenediamine tetra-acetic acid-containing tubes (Vacutainer, SST, Becton Dickinson, Plymouth, UK), and were centrifuged at four thousand revolutions per minute for seven minutes at 4ºC and stored at -80ºC. 1,25(OH)$_2$D plasma levels were measured using a DiaSorinLiaison®immunochemiluminometric analyzer (DiaSorin Ltd, Wokingham, Berkshire,

UK) accordingly to the manufacturer's instructions. S-Klotho plasma levels were determined according to a solid-phase sandwich enzyme-linked immunosorbent assay kit (Demeditec, Kiel, Germany) strictly following the manufacturer's recommendations. The kit uses two types of highly specific antibodies (i.e., purified mouse anti-human Klotho IgG). The optical density was measured at a wavelength of 450 nm ± 2 nm and a standard curve was generated using known antigen concentrations.

Anthropometry and body composition

Participants' body weight and body height were measured to the nearest 0.1 kg and 0.1 cm, respectively, with light clothing and without shoes using a pre-validated SECA scale and stadiometer (model 799, Electronic Column Scale, Hamburg, Germany). Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Body composition analysis was performed using a dual-energy-X-ray absorptiometry scanner (DXA, Discovery Wi, Hologic, Inc., Bedford, MA, USA) with analysis software version APEX 4.0.2, obtaining fat body mass (kg) and lean body mass (kg). A whole-body scan was performed to obtain all parameters following the manufacture´s guidelines. The fat mass index (FMI) and the lean mass index (LMI) were calculated as (fat body mass [kg]/ $height^2$ [m]) and (lean body mass [kg]/ $height^2$ [m]), respectively. Bone mineral density (BMD) was calculated as (bone mineral content [g]/ total bone surface [cm^2]).

Physical activity parameters

Sedentary and physical activity time were objectively assessed by triaxial accelerometry employing a wrist-worn accelerometer (ActiGraph GT3X+, Pensacola, FL, United States) for 7 consecutive days (24 hours/day) [28]. Data were exported and processed using the ActiLife v.6.13.3 software (ActiGraph, Pensacola, FL, United States) and the GGIR package (v.1.5-12, https://cran.r-project.org/web/packages/GGIR/) in R software (v.3.1.2, https://www.cran.r-project.org/) [29,30]. The participants who did not wear the accelerometers for at least 16 hours/day during 4 days (including 1 weekend day) were finally excluded from the analysis.

Dietary intake

A total of three 24-hour dietary recalls were performed in non-consecutive days, obtaining 2 of them on weekdays and 1 on a weekend day. All 24-hour recalls were conducted through face-to-face interviews by qualified and trained research dietitians. The interviews consisted on a

detailed description and assessment of food consumption. A colored photograph guide of different portion sizes of food was used to assist participants in term of estimating the amount of food they consumed [31]. The dietary data were introduced, analyzed and processed in order to obtain energy, macronutrients, and micronutrients content using the EvalFINUT® software (FINUT, Granada, Spain), with the USDA (United States Department of Agriculture) and BEDCA ("Base de Datos Española de Composición de Alimentos") databases.

Statistical analysis

The present study is based on a secondary analysis using baseline data from the FIT-AGEING project, and therefore a specific power calculation was not developed for the present study [28]. Descriptive data are presented as mean and standard deviation. The distribution of all variables was verified with visual inspection of histograms, Q-Q and box plots, and the Shapiro-Wilk test. Comparisons between men and women were performed with independent samples T test. No interaction by sex was observed ($P>0.05$), hence the appropriateness of fitting models for men and women were combined including sex as a covariable.

Simple linear regression model was first used to examine the association of $1,25(OH)_2D$ with S-Klotho plasma levels. Hierarchical regression analyses were subsequently performed in order to check whether $1,25(OH)_2D$ predict S-Klotho plasma levels independently of potential confounders. The entry order of potential confounders in the hierarchical analysis were as follows: age, sex, BMI, FMI, LMI, BMD, total energy intake, vitamin D intake, sedentary and physical activity time. Multiple linear regression analyses were built using the derived confounders.

We analyzed the potential mediating role of body composition, sedentary time, physical activity time, dietary intake, calcium and phosphorus levels in the relationship between $1,25(OH)_2D$ plasma levels and S-Klotho plasma levels [32]. Mediation was estimated using the indirect effect, which indicates changes in the effect of the independent variable on the outcome that can be endorsed to the proposed mediator. Indirect effects (a × b paths) with confidence intervals not including zero are interpreted as statistically significant [33] which could occur regardless of the significance of the total effect (i.e., c path, effect of the independent variable on the dependent variable) and the direct effect (i.e., c' path, effect on the dependent variable when both the independent and the mediator variables are included as independent variables) [32]. This estimation is based on the bootstrapping method, a non-parametric resampling method which estimates the indirect effect through 5,000 bias-corrected bootstrap samples and 95% confidence intervals. If these confidence intervals do not include zero, the indirect effect ab can be considered

as different from this value and therefore the mediation is assumed. To quantify the magnitude of the total effect explained by mediation analysis, we calculated the percentage of mediation ([indirect effect / total effect] × 100) when the total effect was larger than the indirect effect with the same direction [32].

All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS, v. 22.0, IBM SPSS Statistics, IBM Corporation, Armonk, NY, USA), and P-value < 0.05 was deemed significant. The mediation analyses were performed using the PROCESS macro version 3.3 developed by Andrew F. Hayes and the graphical presentations were prepared using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

RESULTS

Table 1 shows the characteristics of the study' sample by sex. Plasma levels of $1,25(OH)_2D$ and S-Klotho were 40.3 ± 5.1 pg/ml, and 775.3 ± 363.7 pg/ml, respectively, with no differences between men and women (all $P \geq 0.3$). Based on a hierarchical regression model, we included age, sex and FMI as potential confounders (Table 2).

Table 1. Descriptive characteristics of participants.

	N	All		N	Men		N	Women	
Age (years)	73	53.7	(5.1)	34	54.6	(5.2)	39	53.0	(5.0)
Anthropometry and body composition parameters									
Weight (kg)	73	75.5	(15.0)	34	87.3	(11.1)	39	65.3	(9.3) *
Height (cm)	73	167.7	(9.8)	34	175.7	(6.5)	39	160.7	(6.1) *
Body mass index (kg/m²)	73	26.7	(3.8)	34	28.3	(3.6)	39	25.3	(3.3) *
Lean mass (kg)	73	43.2	(11.7)	34	53.9	(6.5)	39	34.1	(5.8) *
Lean mass index (kg/m²)	73	15.2	(2.9)	34	17.5	(2.0)	39	13.2	(1.8) *
Fat mass (%)	73	40.1	(8.9)	34	34.7	(8.0)	39	44.5	(7.4) *
Fat mass (kg)	73	30.1	(8.5)	34	30.9	(9.8)	39	29.2	(7.1)
Fat mass index (kg/m²)	73	10.8	(3.1)	34	10.0	(3.2)	39	11.4	(2.9)
Bone mineral density (g/cm2)	73	1.1	(0.1)	34	1.2	(0.1)	39	1.0	(0.1) *
Dietary intake									
Total Energy intake (kcal/day)	72	2071.7	(455.4)	34	2312.1	(402.9)	38	1854.6	(390.3) *
Vitamin D intake (μg/day)	72	5.0	(6.0)	34	3.8	(3.3)	38	6.1	(7.6)
Calcium intake (mg/day)	72	763.4	(340.5)	34	867.3	(396.9)	38	670.5	(251.4) *
Phosphorus intake (mg/day)	72	1324.7	(558.9)	34	1507.6	(689.6)	38	1161.0	(342.2) *
Sedentary behaviour and physical activity levels									
Sedentary time (min/day)	70	745.9	(84.8)	33	770.7	(81.4)	37	723.7	(82.6) *
Total physical activity (min/day)	70	269.5	(75.1)	33	265.2	(79.3)	37	273.3	(72.0)
Blood parameters									
1,25(OH)2D (pg/ml)	73	40.3	(14.1)	34	38.3	(13.4)	39	42.0	(14.6)
S-Klotho (pg/ml)	73	775.3	(363.7)	34	814.1	(452.2)	39	741.4	(265.6)
Calcium (mg/dl)	73	9.86	(0.48)	34	9.81	(0.47)	39	9.90	(0.48)
Phosphorus (mg/dl)	73	3.32	(0.54)	34	3.06	(0.50)	39	3.54	(0.47)

Data are presented as means (standard deviation). *Significant differences between sexes obtained by the independent sample T test (P<0.05). 1,25(OH)₂D: 1,25-dihydroxyvitamin D; S-Klotho: shed form of the Klotho protein.

Table 2. Hierarchical regression between 1,25(OH)₂D and S-Klotho plasma levels.

	β	R² change	Sig. F change	P
S-Klotho plasma levels				
1,25-dihydroxyvitamin D (pg/ml)				
Age (years)	-0.420	0.450	0.001	0.001
Sex	0.326	0.035	0.028	0.013
Body mass index (kg/m²)	2.277	0.019	0.092	0.023
Lean mass index (kg/m²)	-0.884	0.163	0.001	0.239
Fat mass index (kg/m²)	-1.883	0.006	0.216	0.024
Bone mineral density (g/cm²)	-0.178	0.014	0.066	0.075
Total physical activity time (min/day)	-0.170	0.002	0.500	0.248
Sedentary time (min/day)	-0.252	0.009	0.127	0.107
Total energy intake (kcal/day)	0.003	0.009	0.320	0.973
Vitamin D intake (µg/day)	0.068	0.013	0.386	0.383

β: Standardized regression coefficient; R²; and P value were obtained from the hierarchical multiple linear regression analyses.

Simple linear regression analysis showed a weak tendency toward an inverse association between 1,25(OH)₂D and S-Klotho plasma levels (β=-0.222, R²=0.049, P=0.059; Figure 1). The association was partially attenuated after controlling for age and sex (P=0.164 and P=0.074 respectively; Table 3) and become significant after controlling for FMI (P=0.047; Table 3). However, this association disappeared after controlling for age, sex and FMI (P>0.05; Table 3). We also calculated the 1,25(OH)₂D/S-Klotho ratio, which was positively associated with age (β=0.551, R²=0.304, P<0.001; Figure 2).
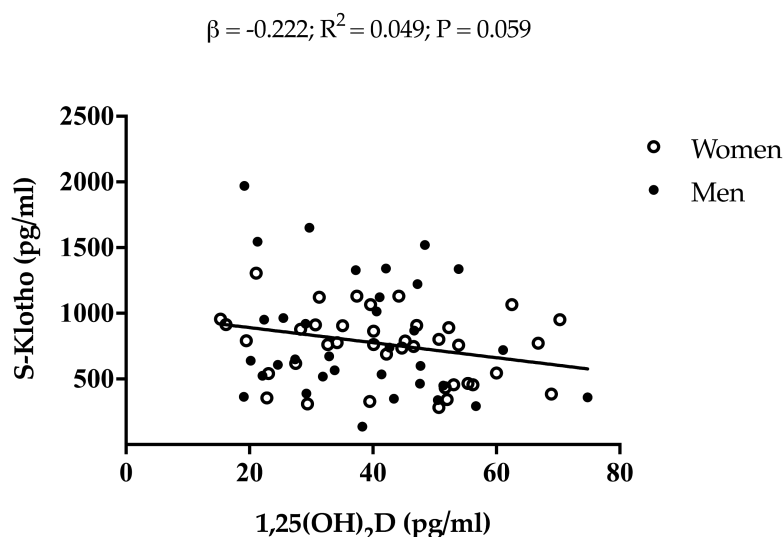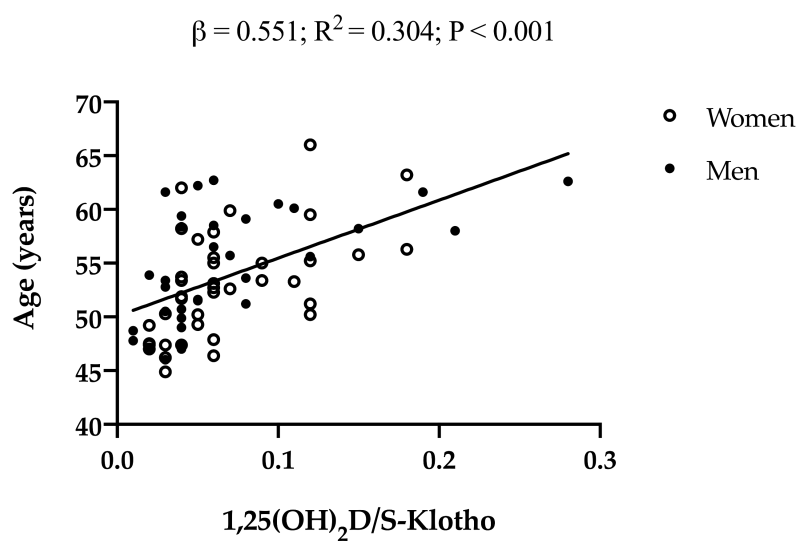
$$\beta = -0.222; R^2 = 0.049; P = 0.059$$

Figure 1. Simple linear regression graph between 1,25-dihydroxyvitamin D (1,25(OH)₂D) and S-Klotho plasma levels in middle-aged sedentary adults. β (standardized regression coefficient), R², and P from a simple linear regression an

Table 3. Multiple linear regression analyses indicating the association of 1,25-Dihydroxyvitamin D with S-Klotho plasma levels.

	S-Klotho plasma levels		
	β	R²	P
1,25-Dihydroxyvitamin D			
Model 1	-0.121	0.493	0.164
Model 2	-0.213	0.055	0.074
Model 3	-0.234	0.071	0.047
Model 4	-0.100	0.536	0.247

The analyses were controlled for: age (Model 1); sex (Model 2); FMI (Model 3); age, sex, and FMI (Model 4). β (standardized regression coefficient); R² and P value were obtained from the multiple linear regression analyses. FMI: Fat mass index.

$$\beta = 0.551; R^2 = 0.304; P < 0.001$$

Figure 2. Simple linear regression graph between age and ratio between 1,25-dihydroxyvitamin D (1,25(OH)₂D) and S-Klotho plasma levels in middle-aged sedentary adults. β (standardized regression coefficient), R², and P from a simple linear regression analysis.

Simple mediation analyses were carried out to test whether the association of 1,25(OH)₂D with S-Klotho plasma levels could be mediated by body composition, sedentary and physical activity time, dietary intake, calcium and phosphorus levels. We observed an inverse association of BMD and S-Klotho levels (b path P=0.015; Figure 3). There was a significant indirect effect (path ab) between 1,25(OH)₂D and S-Klotho levels when BMD was included as a mediator outcome (Figure 3). The association between 1,25(OH)₂D and S-Klotho levels was indirectly influenced by BMD, with a percentage of mediation of 31.40%. Mediation analyses were not significant when other body composition parameters, sedentary and physical activity time, dietary intake, calcium and phosphorus levels were included as mediator variables (Table 4).
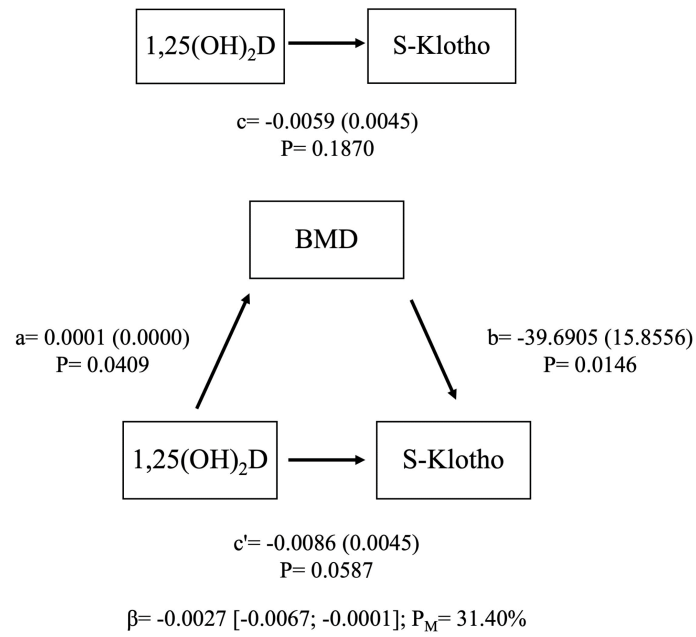
Figure 3. Mediation model of the relationship between 1,25-dihydroxyvitamin D (1,25(OH)₂D) and S-Klotho plasma levels bone mineral density as a mediator variable. Paths a, b, c and c' are presented as unstandardized coefficients (SE). β= indirect effect (a x b paths) [lower-limit CI; upper-limit CI], lower and upper levels for bias-corrected 95% CIs of the indirect effect based on 5,000 bootstraps. 1,25(OH)₂D: 1,25-dihydroxyvitamin D; BMD: Bone mineral density.

Table 4. Total, direct, and indirect effects, a and b pathways, of the simple mediation analyses investigating body composition, sedentary and
parameters as mediator between S-Klotho plasma levels and 1,25-dihydroxyvitamin D (1,25(OH)$_2$D) in middle-aged sedentary adults.

Outcome	Total effect (c)		Direct effect (c')		Path a		Path b		
	Coefficient (SE)	P	Coefficient (SE)	P	Coefficient (SE)	P	Coefficient (SE)	P	C
Body composition									
BMI (kg/m^2)	-0.0086 (0.0045)	0.0587	-0.8312 (0.4539)	0.0713	0.0037 (0.0012)	0.0022	-0.0056 (0.0047)	0.2416	-0
FMI (kg/m^2)	-0.0086 (0.0045)	0.0587	-0.4987 (0.5271)	0.3474	-0.0011 (0.0010)	0.2758	-0.0092 (0.0045)	0.0466	0
LMI (kg/m^2)	-0.0086 (0.0045)	0.0587	-1.0332 (0.6998)	0.1443	0.0046 (0.0008)	<0.0001	-0.0038 (0.0055)	0.4866	-0
Sedentary and physical activity time									
Sedentary time (min/day)	-0.0100 (0.0045)	0.0310	-0.0273 (0.0195)	0.1668	-0.0291 (0.0280)	0.3033	-0.0108 (0.0045)	0.0204	0
Total physical activity (min/day)	-0.0100 (0.0045)	0.0310	0.0014 (0.0224)	0.9495	0.0297 (0.0248)	0.2349	-0.0101 (0.0046)	0.0333	0
Dietary intake									
Total Energy intake (kcal/day)	-0.0088 (0.0045)	0.0546	-0.0017 (0.0035)	0.6314	-0.0417 (0.1563)	0.7904	-0.0089 80.0045)	0.0543	0
Vitamin D intake (μg/day)	-0.0088 (0.0045)	0.0546	0.2380 (0.2856)	0.4075	-0.0049 (0.0019)	0.0115	-0.0076 (0.0047)	0.1108	-0
Calcium intake (mg/day)	-0.0088 (0.0045)	0.0546	-0.0012 (0.0049)	0.8105	0.1181 (0.1103)	0.2882	-0.0086 (0.0046)	0.0623	-0
Phosphorus intake (mg/day)	-0.0088 (0.0045)	0.0546	-0.0007 (0.0030)	0.8259	0.0510 (0.1824)	0.7805	-0.0087 (0.0030)	0.0574	0
Blood parameters									
Calcium (mg/dl)	-0.0086 (0.0045)	0.0587	-0.2834 (3.4624)	0.9350	-0.0001 (0.0002)	0.4773	-0.0087 (0.0045)	0.0605	0
Phosphorus (mg/dl)	-0.0086 (0.0045)	0.0587	1.8969 (3.0446)	0.5353	0.0000 (0.0002)	0.9905	-0.0086 (0.0045)	0.0597	0

Results showed as unstandardized coefficients (standard error, SE) and BC 95% CI based on 5000 bootstraps. BMI: Body mass index; FMI: Fat mass in

DISCUSSION

Our study sought to elucidate the potential association of $1,25(OH)_2D$ with S-Klotho plasma levels and, in turn, whether body composition parameters, physical activity levels, dietary parameters and blood markers were potential mediating mechanisms explaining the closed association between $1,25(OH)_2D$ and S-Klotho in middle-aged sedentary adults. The present results suggest that $1,25(OH)_2D$ is slightly inversely associated with S-Klotho, with this association being increased after adjusting by FMI, and attenuated after adjusting by age and sex. Interestingly, BMD significantly explained the association between these two variables. To the best of our knowledge, this is the first study that elucidates an inverse relationship between $1,25(OH)_2D$ and S-Klotho plasma levels in humans.

Both $1,25(OH)_2D$ and S-Klotho have emerged as important hormones related to the promotion of health span by delaying chronic diseases [34]. Indeed, previous studies have suggested that $1,25(OH)_2D$ and S-Klotho could function in a closed endocrine loop involving several physiological and molecular pathways that may lead to increase health span and prevent chronic diseases [34]. The relationship between $1,25(OH)_2D$ and Klotho has been previously investigated in mice [22-25] showing that a downregulation of α-Klotho gene expression induces an overexpression of 1α-hydroxylase. This fact was explained by an absence of the inhibitory effect of FGF23 signaling on renal 1α-hydroxylase (CYP27B1) expression [25], leading to higher production of $1,25(OH)_2D$ [20,35] which produces subsequent mineral and hormone imbalances related to premature aging syndrome [36]. On the other hand, a recent clinical trial conducted by Azimzadeh et al. showed that 12-weeks of 50.000 IU Vitamin D3 (cholecalciferol) supplementation prevents the reduction of S-Klotho plasma levels inherent to the aging process in older population [37]. Furthermore, this study also observed an increment of 25-hydroxyvitamin D levels after the supplementation. However, the $1,25(OH)_2D$ plasma levels were not determined [37] therefore, it is unknown if changes in $1,25(OH)_2D$ levels occurred. The slightly inverse association between $1,25(OH)_2D$ and S-Klotho plasma levels in our study concurs with those observed in mice models, and could be explained by an inhibitory feedback loop between both metabolites. Higher levels of $1,25(OH)_2D$ could increase S-Klotho plasma levels downregulating $1,25(OH)_2D$ plasma levels in a negative feedback loop [34,38]. Similarly, low levels of S-Klotho could increase the activity of 1α-hydroxylase subsequently increasing $1,25(OH)_2D$ levels [20,25,35]. S-Klotho decrease upon age, inhibiting FGF23 signaling of $1,25(OH)_2D$ inactivation in kidney, being subsequently upregulated [38]. Therefore, since we observed that $1,25(OH)_2D$/S-Klotho ratio was positive associated with age, it could be a plausible biomarker of age.

Our results suggest a mediation role of BMD in the relationship between 1,25(OH)$_2$D and S-Klotho plasma levels. FGF23 is mainly produced in bone by osteoblasts and osteocytes in response to elevated 1,25(OH)$_2$D [39] functioning as an auto-/paracrine inhibitor of bone mineralization by suppressing alkaline phosphatase [40]. In this sense, high FGF23 levels have been associated with an impaired trabecular bone microarchitecture in osteoporosis [41], low BMD in childhood [42], and biomarkers of vertebral fractures in elderly [43]. It seems therefore plausible that the mediation role of the BMD may be explained by FGF23 plasma levels, suggesting the presence of an endocrine bone-kidney axis in humans, where 25(OH)D, 1,25(OH)$_2$D, FGF23 and S-Klotho are involved [38,39] (Figure 4): (i) 1,25(OH)$_2$D would increase intestinal calcium and phosphorus absorption and bone deposition ensuring an adequate BMD status [44], and in a negative feedback loop, it would be suppressed by the restoration of a normal range of calcium and BMD [44]; (ii) 1,25(OH)$_2$D would promote FGF23 secretion and, in a negative feedback loop, 1,25(OH)$_2$D would be suppressed by FGF23 [39]. FGF23 also downregulates the synthesis of 1α-hydroxylase in the renal proximal tubules, thus suppressing 1,25(OH)$_2$D production [39]; (iii) 1,25(OH)$_2$D increases S-Klotho plasma levels and, in a negative feedback loop, 1,25(OH)$_2$D would be suppressed by S-Klotho [34,38]. Furthermore, 1,25(OH)$_2$D is part of the structural basis of the FGF23-α-Klotho-FGFR1c complex formation which triggers into increased levels of S-Klotho [38]; (iv) FGF23 is released by bone cells [39] and is associated with a poor bone status [41–43]. This fact could be explained by the suppression of 1,25(OH)$_2$D. Similarly, given that FGF23 took part of the structural basis of the

FGF23-α-Klotho-FGFR1c complex formation, it may lead to an increased release of S-Klotho into circulation [38]; (v) S-Klotho is associated with higher BMD in middle-aged adults [27].

Taking all together, our results suggest that BMD is closely related to the FGF-Klotho endocrine system, which has been identified as a key factor in the pathophysiology of age-related disorders [38]. Our results have a marked clinical implication, since the disruption of the FGF23-αKlotho endocrine axis has an important role in the pathophysiology of renal and bone disorders [38]. Future studies investigating the
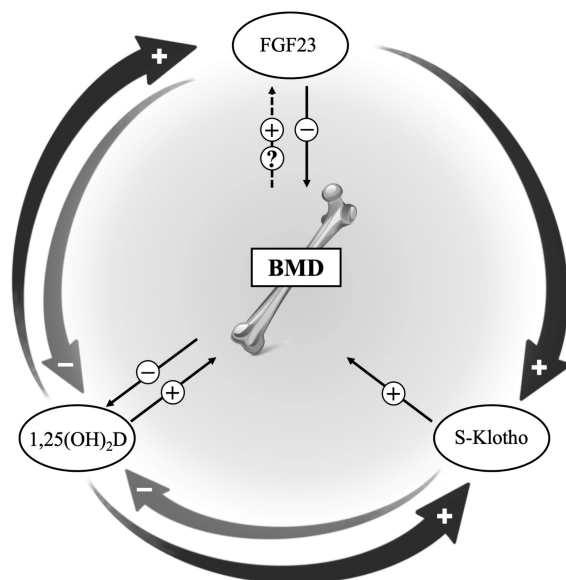

Figure 4. 1,25(OH)$_2$D-bone mineral density-S-Klotho axis. 1,25(OH)$_2$D: 1,25-dihydroxyvitamin D; BMD: Bone mineral density; FGF23: Fibroblast growth factor 23.

influence of different interventions in the whole FGF23-αKlotho endocrine axis where BMD is taking into account are needed.

Limitations

The limitations of the present study include a cross-sectional design, which means that no causal relationships can be established. Our participants were middle-aged sedentary adults (45-65 years old), so we cannot extrapolatable these finding to older, younger, and/or physically active individuals. We did not have data of FGF23 and 25-hydroxyvitamin D plasma levels, which would be desirable to well-understand our study findings. Lastly, since the sample size of this study is relatively small, the data should be interpreted with caution.

CONCLUSIONS

In summary, our results suggest that 1,25(OH)$_2$D is negatively associated with S-Klotho plasma levels in middle-aged sedentary adults, which is partially mediated by BMD. Therefore, we suggest that BMD should be taken into account in future studies investigating the relationship of 1,25(OH)$_2$D with S-Klotho plasma levels. Future studies are therefore needed to elucidate whether changes in 1,25(OH)$_2$D and/or BMD are related to changes in S-Klotho plasma levels after different anti-aging interventions (i.e., vitamin D supplementation, exercise and/or dietary interventions).

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SECTION 2

Role of exercise on 1,25-dihydroxyvitamin D

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Chapter 7

1,25-dihydroxyvitamin D is increased in response to different exercise training interventions in healthy sedentary adults: the FIT-AGEING randomized controlled trial **(Study 5)**

ABSTRACT

Background & aims: Vitamin D deficiency is currently endemic worldwide and is considered as an important factor in the development of several chronic conditions. Physical exercise has been postulated as an auspicious strategy to counteract age-related disorders preventing premature mortality. However, the effect of chronic exercise training on 1,25-dihydroxyvitamin D [1,25(OH)$_2$D] is unclear. This 12-week randomized controlled trial aimed to investigate (i) the effects of different training modalities on 1,25(OH)$_2$D in healthy sedentary adults, and (ii) whether these hypothetical changes in 1,25(OH)$_2$D are associated with changes in body composition and physical fitness

Methods: A total of 89 healthy sedentary adults (52.7% women; 53.5 ± 4.9 years old) were enrolled in the FIT-AGEING study. The participants were randomized to: (i) a control group (no exercise); (ii) physical activity recommendation from the World Health Organization (PAR group); (iii) high-intensity interval training (HIIT group); and (iv) HIIT adding whole-body electromyostimulation training (HIIT+EMS). 1,25(OH)$_2$D plasma levels were measured using a DiaSorin Liaison® immunochemiluminometric analyzer. Body composition was determined by dual X-ray absorptiometry. Cardiorespiratory fitness was determined by a maximum treadmill test using indirect calorimetry. Lower and upper muscular strength was assessed by an isokinetic strength test and by the handgrip strength test, respectively

Results: Compared to the control group, 1,25(OH)$_2$D increased in PAR (Δ=10.99 ± 3.44 pg/ml; P=0.013), HIIT (Δ=11.63 ± 3.51 pg/ml; P=0.009), and HIIT+EMS groups (Δ=14.01 ± 3.59 pg/ml; P=0.001) without statistical differences between them (all P>0.1). No association was found between changes in body composition and changes in 1,25(OH)$_2$D (all P>0.1). A significant positive association was found between changes in both maximum oxygen uptake, extension peak torque, and hand grip strength and 1,25(OH)$_2$D (all P< 0.01).

Conclusions: In summary, our results show that a 12-week exercise intervention produced an increment of 1,25(OH)$_2$D independently of age, sex and exercise modality in healthy sedentary adults. Furthermore, we also found a significant positive association between changes in physical fitness and changes in 1,25(OH)$_2$D in our study cohort. Therefore, we suggest that the link between an exercise intervention and the increase of physical fitness could be in part mediated by changes in vitamin D metabolism.

BACKGROUND

Vitamin D deficiency is currently endemic worldwide, not only in undeveloped countries but also in developing and developed countries [1]. Indeed, this metabolic problem affects all age groups – especially elderly adults – and its incidence is independent of the individual' sex [1]. Previous studies have reported that the vitamin D status plays a key role in the ageing process additionally to its important implications on bone and skeletal muscle metabolism [2]. Given that vitamin D receptors have been found in more than thirty different types of cells, it has been suggested that vitamin D also exerts additional functions in further tissues and organs [3]. This argument is supported by the notion that poor levels of vitamin D have been associated with increased prevalence of several age-related diseases and mortality in both healthy individuals and patients [4,5]. Serum 25-hydroxyvitamin D levels [25(OH)D] have traditionally been used as an indicator of vitamin D status. However, considerably less attention has been paid to 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] (i.e., calcitriol) which is the principal responsible of its metabolic properties [1,6].

In addition to medical treatment, physical exercise has been postulated as an auspicious strategy to counteract both mental and physical chronic disorders [7]. Concretely, Pedersen and Saltin proposed physical exercise as the most important instrument to prevent and treat more than thirty-five chronic diseases (e.g., psychiatric disorders, metabolic pathologies, cardiovascular diseases, or cancer) [8]. Moreover, a 30% reduced risk of mortality can be attained when a well-designed and structured physical exercise program is performed, thus increasing longevity and quality of life during the aging process [9].

Previous scientific evidence has demonstrated the efficacy of physical exercise to prevent and/or delay the deleterious effects of the aging process on physiological functions [10]. Nevertheless, it remains partially unknown the molecular and biological pathways that explain the exercise-related positive influence on human' health in old age. In this regard, considerably little attention has been focused upon physical exercise as a regulator of vitamin D status. Interestingly, it has been reported that an aerobic training intervention ranged from 5 to 8 weeks induced a significant increment of 25(OH)D in elderly adults [11,12]. The problem of studying ageing metabolic biomarkers in old individuals is that most of them suffer from age-related illnesses [13]. From a preventive perspective, it is, therefore, of scientific and clinical interest to study these metabolites in relatively young individuals free of chronic disorders [14]. To the best of our knowledge, there is no study investigating the effects of physical exercise on the active form of vitamin D. Furthermore, no data are yet available concerning whether different exercise training

modalities could induce contrasting effects on 1,25(OH)$_2$D. Therefore, this study aimed to investigate the effects of different exercise training modalities on 1,25(OH)$_2$D in healthy sedentary adults. A further aim of this study was to determine whether changes in other health-related parameters (i.e., body composition and physical fitness) were associated with these hypothetical changes in 1,25(OH)$_2$D in our study' cohort.

MATERIALS AND METHODS

Participants

Eighty-nine healthy sedentary adults (52.7% women) were voluntarily enrolled in the FIT-AGEING study, an exercise-based randomized controlled trial (clinicaltrial.gov: ID: NCT03334357) [15]. Figure 1 shows the flowchart of this study. The participants were recruited from the province of Granada (Spain) via local media, social networks, and posters. Interested individuals were screened via telephone and/or e-mail. A research medical staff conducted a medical examination to determine whether potentially eligible participants met the following inclusion criteria: (i) adults aged between 45 and 65 years old, (ii) to be sedentary (less than 20 minutes of moderate-intensity physical activity on 3 days/week over the last three months), and (iii) to have a stable weight over the last three months. The exclusion criteria were as follow: (i) suffering from chronic cardiometabolic diseases, (ii) taking any medication, and (iii) having a major illness (acute or chronic) including any that would limit the ability to perform the necessary exercises. The study protocols and experimental design followed the principles of the last revised Declaration of Helsinki [16] and was approved by the Ethics Committee on Human Research of the Regional Government of Andalucía [0838-N-2017]. All participants provided oral and written informed consent after having read and understood the details of the exercise programs and the experimental procedures. All of the baseline and follow-up examinations were performed in the same setting [*Centro de Investigación Deporte y Salud* (*CIDS*, Granada, Spain) and at the "Campus de la Salud" Hospital (Granada, Spain)].

Study design

A randomized controlled trial with a parallel-group design was performed over 12 weeks according to the CONSORT statement for transparent reporting [17]. The RCT was conducted in two waves (September–December 2016 and September-December 2017) of forty-five participants maximum. After the baseline assessment was performed, participants were linked to a study

identification number and were then randomized into four different groups using a computer-generated simple randomization software [18] in the following groups: (i) a control group (no exercise), (ii) a concurrent training based on physical activity recommendation from the World Health Organization (PAR) group, (iii) a high-intensity interval training (HIIT) group, and (iv) a high-intensity interval training adding whole-body electromyostimulation (HIIT+EMS) group. The assessment staff was blinded to the group allocation.

Exercise training modalities

A full description of each exercise training modality has been previously published [15].

Briefly, the participants allocated to the PAR group completed 3 concurrent training (i.e., aerobic plus resistance training) sessions per week for 12 consecutive weeks with at least 48 hours of recovery between sessions. This training program was based on the minimum physical activity recommended by the World Health Organization [19]. The training volume was 150 min/week at an intensity of 60-65% of the heart rate reserve for the aerobic training, while ~60 min/week at an intensity of 40-50% of one-repetition maximum was established for the resistant training. The aerobic training section was performed using a treadmill, cycle-ergometer, and elliptical ergometers. For the resistance training section, weight-bearing and guided pneumatic machines were used. To reduce the risk of injuries as well as to promote exercise adherence, compensatory exercises (i.e., flexibility, stabilizer muscles, and core stability) were included.

The participants allocated into the HIIT group completed 2 sessions per week for 12 weeks with at least 72 hours of recovery between sessions. This training program involved two different and alternative high-intensity interval training protocols [20,21], which included a high intensity interval training with long intervals (type A session), and a high-intensity interval training with short intervals (type B sessions). Type A session was characterized by a training volume of 40-65 min/week at >95% of the maximum oxygen uptake (VO_2max). The participants completed type A session walking on a treadmill with a personalized slope. Type B session was composed by a training volume of 40-65 min/week at level 6-9 on a perceived maximum effort scale [ranged from 0 to 10] [22]. Eight weight-bearing programmed exercises in a circuit form (i.e., squat, deadlift, high knees up, high heels up, push up, horizontal row, lateral plank, and frontal plank) were performed in type B sessions.

The participants allocated in the HIIT+EMS group completed a training program following the same methodology that the HIIT group (i.e., periodization, training frequency, volume, intensity, and type of exercise) combined with electrical pulses with a whole-body

electromyostimulation wireless device (Wiemspro®, Malaga, Spain). The programmed electric pulse was characterized by: (i) a frequency of 15-20 Hz, an intensity of 100 mA, an impulse breadth of 200-400 μs, and a duty cycle of 99% in the type A sessions; and (ii) a frequency of 35-75 Hz, an intensity of 80 mA, an impulse breadth of 200-400 μs, and a duty cycle of 50-63% in the type B sessions.

All training sessions were supervised by a qualified and certified graduate in Sport Sciences, who constantly motivated the participants and instructed them to reach the specific target intensity in all sessions. Before each training session, all participants underwent a dynamic standardized warm-up including general mobility exercises. Training sessions concluded with a cooling-down protocol (i.e., active global stretching). Heart rate was continuously monitored during exercise and the rated perceived exertion scale was also registered in all sessions. Furthermore, a gradual progression was also proposed to control the exercise dose in each training group [15]. To be included in the final analysis, participants were required to attend at least 90% of sessions.

The participants of the control group were instructed to maintain their lifestyle and not being enrolled in any structured exercise program during the intervention. For ethical reasons, participants were invited to an information meeting where the research staff provided general advice about a healthy lifestyle.

Blood sample collection and 1,25-dihydroxyvitamin D assessment

A 10 mL peripheral blood samples were collected using the Vacutainer SST system (Becton Dickinson, Plymouth, UK). They were obtained from an antecubital vein of the forearm after a 12-hour overnight fast, and then centrifuged at 4,000 rpm for 7 minutes at 4ºC. All samples were collected at baseline and after the 12-week intervention. Aliquots of plasma were stored at -80ºC until further analysis. $1,25(OH)_2D$ plasma levels were determined using a DiaSorin Liaison® immunochemiluminometric analyzer (DiaSorin Ltd, Wokingham, Berkshire, UK) and expressed as picograms per milliliter. All participants were previously requested to abstain from drugs, alcohol, and/or caffeine, to eat a standardized dinner, and to avoid any physical activity of moderate (24 h before) and/or vigorous intensity (48 h before). Blood samples were obtained after 72–96 h of the last bout of exercise in the post-intervention assessment.

Anthropometric and body composition assessment

Anthropometric and body composition measurements were performed before and after the intervention program. Height and body mass were measured through a pre-validated Seca model 799 scale and stadiometer (Seca, Hamburg, Germany) with light clothing and without shoes. Body mass index (BMI) was subsequently calculated by dividing body mass (kg) by the squared height (m^2). Lean mass, fat mass, and bone mineral density assessment were performed by dual X-ray absorptiometry (Discovery Wi, Hologic, Inc., Bedford, MA, USA) under the manufacture's recommendations. Fat mass was also expressed as a percentage of the total body mass.

Cardiorespiratory fitness assessment

VO$_2$max was measured through a maximum treadmill (H/P/Cosmos Pulsar treadmill, H/P/Cosmos Sport & Medical GMBH, Germany) exercise test applying the modified Balke protocol [23]. Briefly, the incremental protocol began walking at 3km/h at 0% grade for the first minute followed by two minutes at 4km/h. Treadmill speed was increased to 5.3 km/h at 0% grade for 1 min with subsequent increases of 1% of the grade every minute until the participants reached their volitional exhaustion. Gas exchange was continuously measured (CPX Ultima CardiO2, Medical Graphics Corp, St Paul, USA). O$_2$ uptake and CO$_2$ production were averaged every 5 seconds with the Breeze Suite Software (version 8.1.0.54 SP7, MGC Diagnostic®, ST Pau, MN, USA). Flow calibration was performed by a 3-L syringe, while gas calibration was performed with two standard gas concentrations prior to each use according to the manufacturer's instructions. The 6-20 Borg scale rating of perceived exertion was applied during the last 15 seconds of each stage and at exhaustion, while heart rate was measured continuously (every 5 seconds) (Polar RS300, Kempele, Finland). VO$_2$max was defined by the following criteria: (i) to attain a respiratory exchange ratio ≥ 1.1, (ii) to show a plateau in the VO$_2$ curve despite increasing the exercise intensity (defined as a change of <100 ml/min in the last 30s), and (iii) to reach the age-predicted maximal heart rate (209-0.73 * age) ± 10 beats/min [24]. Peak oxygen consumption value was considered when these criteria were not met [25]. The participants were previously instructed to meet the following pre-conditions: (i) to refrain from stimulant substances at least 24 hours before the test, (ii) to fast for 3 hours, and (iii) to avoid any physical activity of moderate and/or vigorous intensity for 24/48 hours before the test, respectively.

Muscular strength assessment

Isokinetic knee extension strength was measured with a Gymnex Iso-2 dynamometer (Easytech s.r.l. Italy) applying the same preconditions as in the cardiorespiratory fitness measurement. The isokinetic dynamometer was calibrated following the manufacturer´s instructions prior to starting the data collection. The isokinetic peak torque of extensor muscles was measured at an angular velocity of 60°/s. The participants were positioned on the seat, securing the upper leg, hips, and shoulder to the chair using safety belts ensuring any extra movement of the body during the test. For safety reasons, a knee joint of motion angle ranged from 90º to 170º was set for each participant. All of them performed five submaximal repetitions (i.e., familiarization protocol), followed by a 1-minute rest interval. Then, three maximal repetitions were completed to evaluate the isokinetic knee strength [26]. The peak concentric torque (Nm) was determined as the single repetition with the highest muscular force output. Constant verbal motivation encouragement was given to participants during the tests to generate maximum effort in each repetition, and the same trained researcher conducted all the isokinetic tests.

Handgrip strength was measured using a digital hand dynamometer with an adjustable grip (T.K.K. 5401 Grip-D; Takey, Tokyo, Japan). The participants alternately completed two attempts with each hand, and were asked to generate the maximum isometric force during 2-3 s. Following previous studies, the optimal grip span of the dynamometer was adjusted at 5.5 cm for men, and calculated with a validated equation for women [27]. We considered total handgrip strength as the sum of the highest value (in kg) of each hand.

Dietary intake assessment

Dietary intake was obtained by using the average of three 24-hour food records. They were obtained during an interview performed by a qualified and experimented research dietitian on nonconsecutive days (1 day on the weekend). The recalls were processed on the EvalFINUT® software, which is based on US Department of Agriculture and 'Base de Datos Española de Composición de Alimentos' databases, to obtain data related to energy, macronutrients and micronutrients intake. The interviews were supported by the use of a guide with colored photographs of different food portions sizes in order to facilitate the estimation of the quantity of food consumed [28].

Statistical analysis

The sample size and power calculations were based on a pilot sample (n = 30). A minimum predicted change in $1,25(OH)_2D$ of 10% between the intervention groups and the control group with a standard deviation of 10%. A sample size of 16 participants per group was predicted to provide a statistical power of 85% considering a type 1 error = 0.05 [29]. A minimum of 20 participants per group were recruited assuming a maximum loss at follow-up of 25%.

Data normality was checked using the Shapiro-Wilk test, visual check of histograms, and Q-Q plots. The descriptive parameters are reported as mean and standard deviation. Student's t-tests for unpaired values were conducted to examine differences in dependent variables at the baseline between groups.

Analysis of covariance (ANCOVA) was performed to study the effect of the groups (fixed factor) on dependent outcomes, adjusting for the baseline values (i.e., post-$1,25(OH)_2D$ minus pre-$1,25(OH)_2D$). ANCOVA was also conducted to investigate whether the above-mentioned changes were independent of sex and age and energy/macronutrient intake. We performed Bonferroni post hoc test with adjustment for multiple comparisons to determine differences between all exercise training modalities groups.

To examine the relationship of changes in body composition variables (i.e., body mass, lean mass, fat mass percentage) and physical fitness variables (i.e., VO_2max in absolute values and in relation to body mass, extension peak torque, and hand grip strength) with changes in $1,25(OH)_2D$, we conducted simple linear regression. Multiple linear regressions were also performed adjust by sex (model 1) and age (model 2).

The level of significance was set at $P \leq 0.05$. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, v. 22.0, IBM SPSS Statistics, IBM Corporation). Graphical presentations were prepared using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Figure 1 shows the flowchart for enrolment and analysis. A total of 66 participants (n = 15 in the control group, n = 17 in the PAR group, n = 16 in the HIIT group, and n = 18 in the HIIT+EMS group) completed the study obtaining a global attendance of 99% to the supervised exercised sessions in the intervention groups.
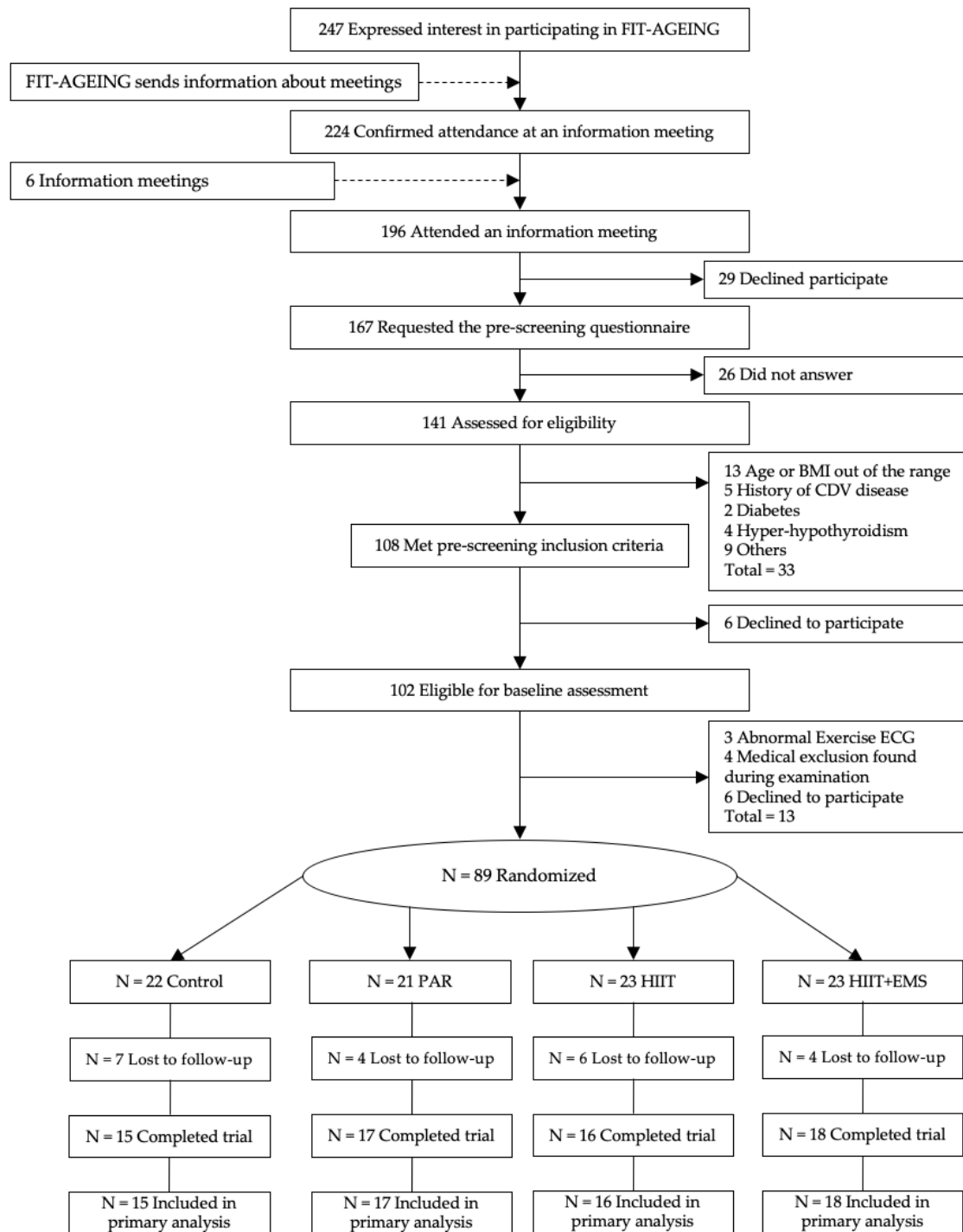
Enrollment and analysis flowchart

Figure 1: Flow-chart diagram. BMI: Body mass index, CDV: Cardiovascular, ECG: Electrocardiogram, PAR: Concurrent training based on physical activity recommendation from the World Health Organization, HIIT: High Intensity Interval Training group, HIIT-EMS: High Intensity Interval Training adding Whole-Body Electromyostimulation group.

Table 1 shows the descriptive characteristics of the study participants at baseline. No significant differences at the baseline were showed in age, sex, body composition, blood parameters, physical fitness, or dietary intake. There were a roughly equal number of men and women in each group.

Table 1. Descriptive baseline parameters.

	All (n=66)	Control (n=15)	PAR (n=17)	HIIT (n=16)	HIIT+EMS (n=18)
Age (years)	53.5 ± 4.9	51.7 ± 4.1	54.9 ± 4.5	53.9 ± 5.5	53.3 ± 5.3
Sex (%)					
Men	30 (45.5)	6 (40.0)	8 (47.1)	7 (43.8)	9 (50.0)
Women	36 (54.5)	9 (60.0)	9 (52.9)	9 (56.3)	9 (50.0)
1,25-(OH)$_2$D (pg/ml)	40.1 ± 13.7	45.5 ± 13.8	42.5 ± 11.6	40.5 ± 9.6	32.8 ± 16.2
Anthropometry and body composition					
Body mass (kg)	75.8 ± 14.9	74.1 ± 13.8	72.6 ± 11.3	76.3 ± 17.9	79.8 ± 16.3
Body mass index (kg/m^2)	26.7 ± 3.9	26.7 ± 3.9	25.4 ± 2.9	26.1 ± 3.2	28.5 ± 4.7
Lean mass (kg)	43.7 ± 11.3	44.3 ± 11.6	43.6 ± 10.8	42.3 ± 12.7	44.7 ± 11.1
Fat mass (%)	39.5 ± 8.5	37.7 ± 8.2	37.4 ± 8.8	42.0 ± 8.1	40.8 ± 8.8
Bone mineral density (g/cm^2)	1.10 ± 0.10	1.10 ± 0.12	1.08 ± 0.08	1.10 ± 0.10	1.12 ± 0.10
Physical fitness					
VO$_2$max (ml/min)	2307.2 ± 623.4	2203.0 ± 610.9	2320.4 ± 649.7	2313.9 ± 599.7	2375.5 ± 670.4
VO$_2$max (ml/kg/min)	30.4 ± 5.4	29.5 ± 4.8	31.6 ± 6.1	30.6 ± 5.9	29.6 ± 5.0
Hand grip strength (kg)	70.6 ± 23.7	70.1 ± 24.4	72.0 ± 25.0	68.3 ± 26.7	71.7 ± 21.0
Extension peak torque (Nm)	267.4 ± 82.6	268.6 ± 74.4	271.5 ± 76.1	285.1 ± 109.2	245.5 ± 67.9
Dietary intake					
Energy intake (kcal/day)	2071.7 ± 455.4	2023.9 ± 497.6	2008.1 ± 385.3	2128.7 ± 545.5	2117.5 ± 428.8
Carbohydrate intake (g/day)	215.3 ± 59.0	217.7 ± 87.3	206.0 ± 40.3	226.0 ± 53.9	212.8 ± 53.9
Fat intake (g/day)	87.9 ± 23.6	86.4 ± 18.7	85.3 ± 27.0	87.3 ± 28.9	92.0 ± 20.1
Protein intake (g/day)	83.0 ± 25.2	72.2 ± 17.7	84.7 ± 32.0	86.3 ± 26.3	87.3 ± 21.6

Data are shown as means ± standard deviation otherwise is stated. PAR: Concurrent training based on physical activity recommendation from the World Health Organization, HIIT: High Intensity Interval Training group, HIIT-EMS: High Intensity Interval Training adding Whole-Body Electromyostimulation group; 1,25(OH)$_2$D: 1,25-dihydroxyvitamin D; VO$_2$max: Maximum oxygen uptake.

Figure 2 shows changes in 1,25(OH)$_2$D after the intervention study among the four groups. Compared to the control group, 1,25(OH)$_2$D increased in PAR (Δ=10.99 ± 3.44 pg/ml; P=0.013), HIIT (Δ=11.63 ± 3.51 pg/ml; P=0.009), and HIIT+EMS groups (Δ=14.01 ± 3.59 pg/ml; P=0.001) without statistical differences between them (all P>0.1, Figure 2). All results persisted after including sex, age, and energy/macronutrients intake as covariates (all P<0.02).
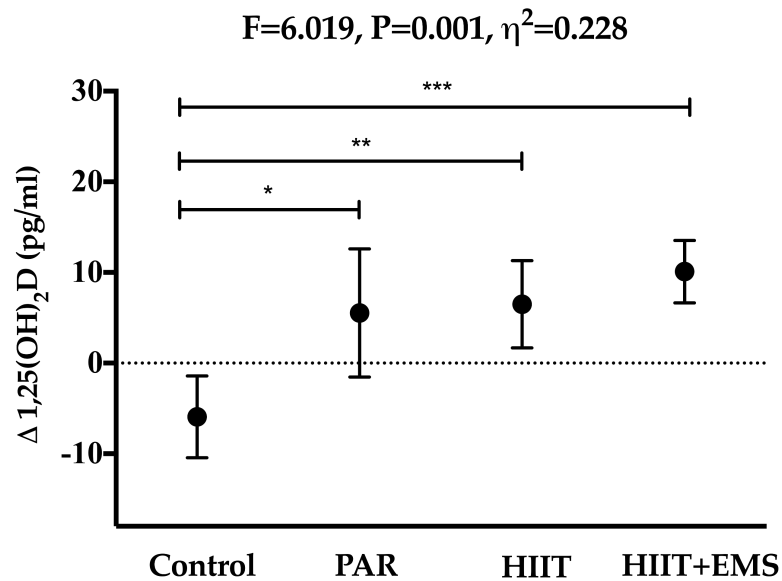
$$F=6.019, P=0.001, \eta^2=0.228$$

Figure 2. Changes in 1,25(OH)$_2$D after the intervention study in the 4 groups. Parallel bars indicate significant differences between groups. *$P<0.05$, **$P<0.01$, ***$P<0.001$, analysis of covariance adjusting for baseline values, with post hoc Bonferroni-corrected t-test. The data are shown as means ± standard deviation. 1,25(OH)$_2$D: 1,25-dihydroxyvitamin D; PAR: Concurrent training based on physical activity recommendation from the World Health Organization; HIIT: High Intensity Interval Training group; HIIT-EMS: High Intensity Interval Training adding Whole-Body Electromyostimulation group.

Figure 3 shows the association between changes in body composition variables and changes in 1,25(OH)$_2$D after the intervention programs. No association was found between changes in body mass, lean mass, fat mass percentage, and bone mineral density with changes in 1,25(OH)$_2$D (all $P>0.1$, Figure 3), which remained after adjusting for sex and age (all $P>0.1$, Table 2).

Figure 4 shows the association between changes in physical fitness variables and changes in 1,25(OH)$_2$D after the intervention programs. A significant positive association was found between the changes in VO$_2$max (in absolute values and in relation to body mass) and changes in the 1,25(OH)$_2$D ($\beta=0.325$, $R^2=0.105$, $P=0.005$, and $\beta=0.324$, $R^2=0.106$, $P=0.008$; Figure 4A and 4B, respectively). Similarly, a significant positive association was found between changes in both extension peak torque and hand grip strength, and changes in the 1,25(OH)$_2$D ($\beta=0.327$, $R^2=0.107$, $P=0.008$, and $\beta=0.378$, $R^2=0.143$, $P=0.002$; Figure 4C and 4D, respectively). All associations remained after including sex and age as covariates (all $P\leq0.015$, Table 2).
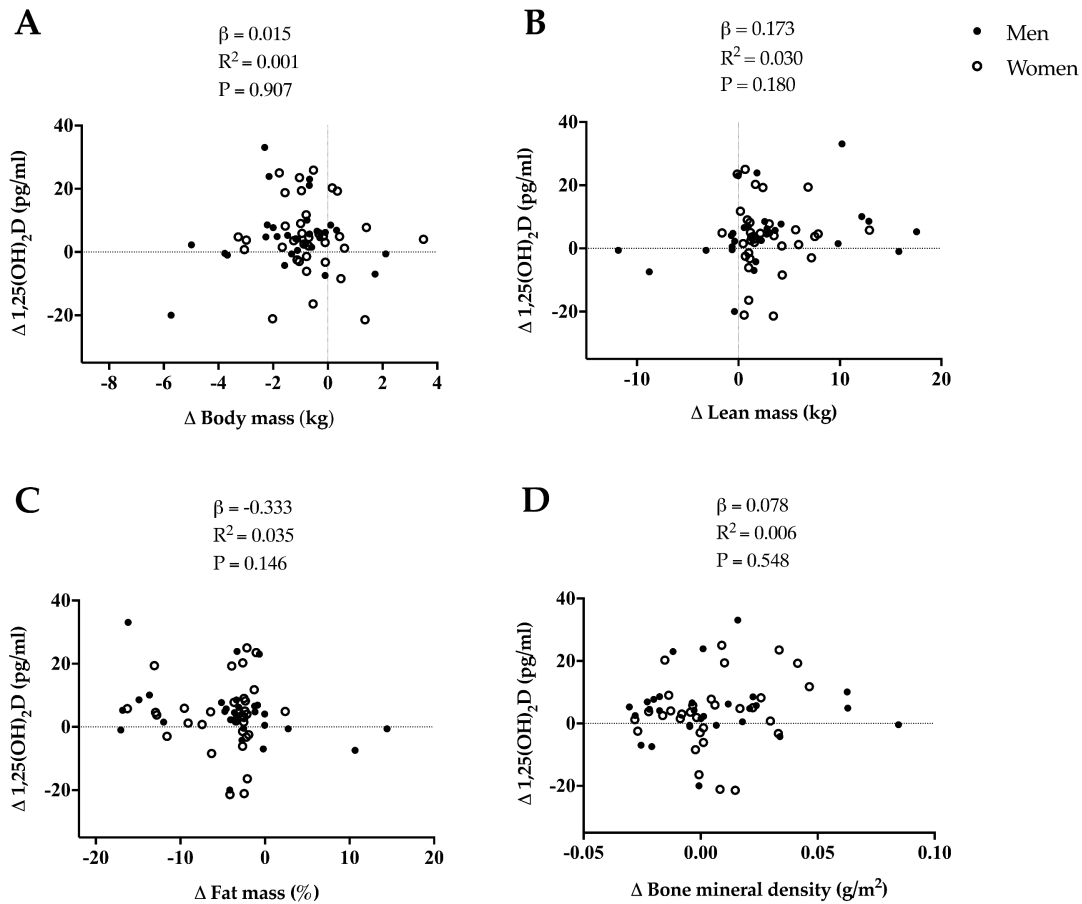
Figure 3. Association between changes in body composition variables which include body mass (Figure 3A), lean mass (Figure 3B), fat mass percentage (Figure 3C) and bone mineral density (Figure 3D) with changes in 1,25(OH)₂D after the intervention program in healthy sedentary adults. β (standardized regression coefficient), R^2, and P from a simple linear regression analysis. 1,25(OH)₂D: 1,25-dihydroxyvitamin D.

Table 2. Association between changes in body mass, lean mass, fat mass percentage, maximum oxygen uptake (VO₂max) in absolute values and in relation to body mass, hand grip strength, and extension peak torque with 1,25-dihydroxyvitamin D changes unadjusted (Model 0), adjusted for sex (Model 1), and adjusted for age (Model 2).

| | Δ 1,25-dihydroxyvitamin D (pg/ml) | | | | | |
| | Model 0 | | Model 1 | | Model 2 | |
	P value	β	P value	β	P value	β
Δ Body mass (kg)	0.907	0.015	0.866	0.022	0.960	0.007
Δ Lean mass (kg)	0.180	0.173	0.182	0.173	0.221	0.165
Δ Fat mass (%)	0.146	-0.187	0.139	-0.192	0.178	-0.180
Δ VO₂max (ml/min)	**0.008**	**0.325**	**0.009**	**0.327**	**0.011**	**0.328**
Δ VO₂max (ml/kg/min)	**0.009**	**0.324**	**0.010**	**0.323**	**0.011**	**0.322**
Δ Hand grip strength (kg)	**0.002**	**0.378**	**0.002**	**0.384**	**0.002**	**0.387**
Δ Extension peak torque (Nm)	**0.008**	**0.327**	**0.009**	**0.331**	**0.010**	**0.332**

P value of multiple-regression analysis. β (standardized regression coefficient). Values in bold indicate significance differences (P<0.05).
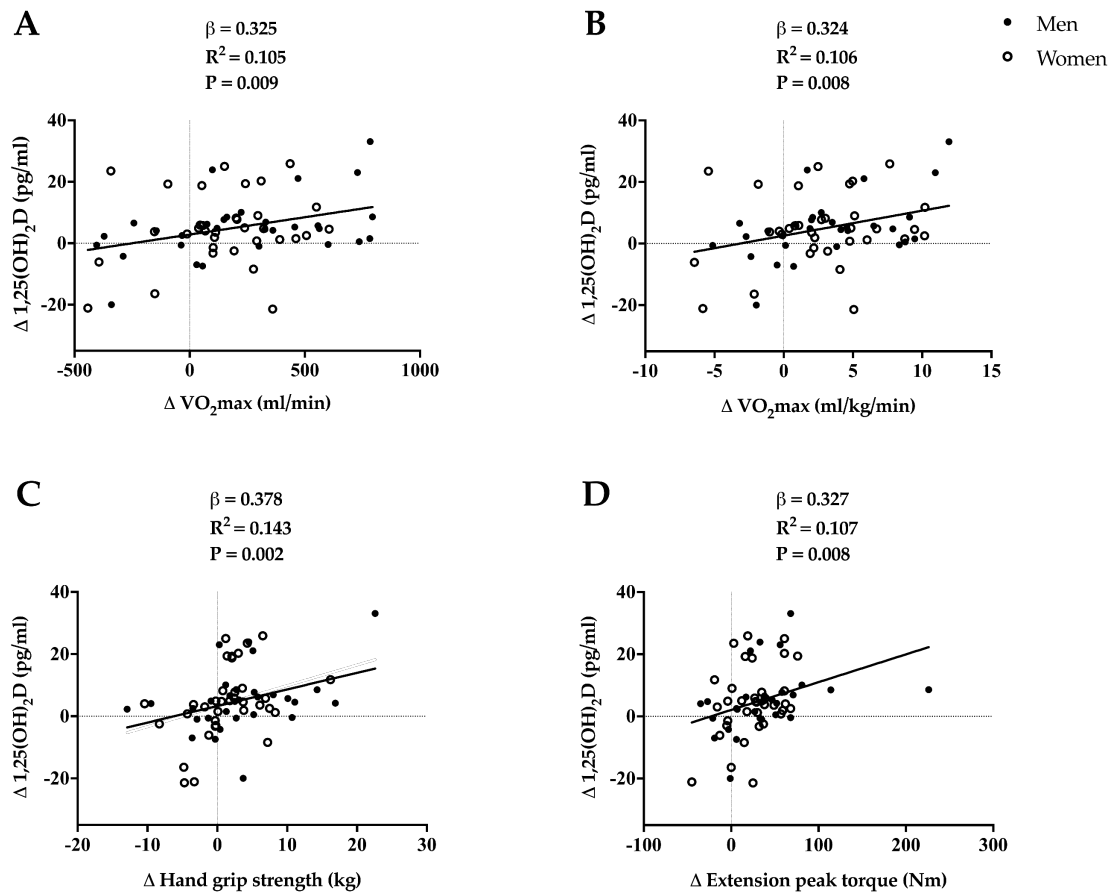
Figure 4. Association between changes in physical fitness variables which include maximum oxygen uptake in absolute values (Figure 4A), and in relation to body mass (Figure 4B), hand grip strength (Figure 4C), and extension peak torque (Figure 4D) with changes in 1,25-dihydroxyvitamin D after the intervention program in healthy sedentary adults. β (standardized regression coefficient), R^2, and P from a simple linear regression analysis. 1,25(OH)$_2$D: 1,25-dihydroxyvitamin D; VO$_2$max (maximum oxygen uptake).

DISCUSSION

The current study sought to elucidate (i) the effects of 3 different exercise training interventions on 1,25(OH)$_2$D in healthy sedentary adults, and (ii) whether exercise-related changes in body composition and physical fitness were associated with changes in 1,25(OH)$_2$D in our study' cohort. The main findings of the present work were that, compared to the control group, the participants included in the intervention groups benefited from a significant improvement in 1,25(OH)$_2$D independently of the exercise modality. Interestingly, while no significant association between changes in body composition and changes in 1,25(OH)$_2$D was observed, a significant positive association was found between changes in physical fitness and changes in 1,25(OH)$_2$D. Taking all together, these results suggest that physical exercise is an effective strategy to increase

1,25(OH)$_2$D in healthy sedentary adults, and that these training effects may partially explain changes in physical fitness.

Three main physiological mechanisms have been postulated for exercise-mediated improvement of vitamin D status [12]. The first one is that white adipocytes are considered a reservoir of 25(OH)D since these cells can uptake 25(OH)D from the bloodstream [30]. It seems therefore plausible that a fat-loss exercise intervention produces an increment of 25(OH)D availability. Indeed, Rock et al. reported that weight loss (presumably accompanied by a decrease in fat mass) was associated with higher serum 25(OH)D in overweight and obese women [30]. Furthermore, skeletal muscle myocytes – in addition to their traditional physiological functions – also act as a 25(OH)D store [31]. Given that chronic physical exercise could promote subsequent increments of skeletal muscle mass, a greater pool of 25(OH)D would be available to be released into peripheral circulation [31]. Lastly, previous scientific studies demonstrated that physical exercise not only optimizes the synthesis and release of 25(OH)D from the liver (also decreasing its breakdown), but also its hydroxylation in the kidney resulting in greater 1,25(OH)$_2$D levels in animal models [32]. However, there is limited evidence regarding the effects of physical exercise on vitamin D metabolism in humans.

Aerobic exercise has been demonstrated to be an effective stimulus to increase 25(OH)D levels after an acute bout of exercise [33] and at the end of a chronic exercise intervention [11,12,34]. On one hand, Sun et al. observed a direct effect of an acute bout of aerobic exercise on the increase in serum 25(OH)D concentrations which persisted until 24h after exercise [33]. On the other hand, significant increments of 25(OH)D were noted in response to an aerobic exercise intervention (ranging from 5 to 12 weeks) in pregnant women [34] and elderly adults [11,12]. However, while Vainionpää et al. did not find any significant change in 25(OH)D after a 1-year aerobic exercise intervention in women free of disease [35], Evans et al. showed a significant decrease of 25(OH)D in response to a 16-week military training intervention in young adults [36]. These discrepancies could be explained by different facts including (a) the individual' age, sex or health status, (b) the analytical procedures to determine 25(OH)D, (c) the different 25(OH)D pre-test values, (d) and, in some cases [36], the lack of a control group which makes unclear whether the obtained changes in 25(OH)Dare explained by the exercise intervention or by seasonal variation which implies different sunlight exposure and dietary habits [37]. However, to the author knowledge, there is no study investigating the effects of exercise on 1,25(OH)$_2$D. We showed, for the first time, that a well-designed and structured 12-week exercise intervention induces significant increments of 1,25(OH)$_2$D in healthy sedentary adults independently of sex, age and exercise training modality which partially support the above-mentioned findings.

A poor vitamin D status has been consistently associated with an increased incidence of chronic metabolic and cardiovascular diseases [38]. Low physical fitness levels and an altered body composition status have been also related to a higher incidence of age-related chronic pathological conditions and all-cause mortality [39,40]. On the one hand, physical fitness depends on different physiological parameters including arterial oxygen availability, muscle contractibility, cardiac output, shunting of blood to myocytes, and oxygen extraction performed by these myocytes among others, all of them improved by physical exercise and modulated by vitamin D metabolites [41]. On the other hand, it is well-known that individuals with sarcopenic obesity (i.e., high fat mass and low lean mass) present reduced 25(OH)D levels not only because a large fat body mass surface provides superior distribution space for fat-soluble compounds, but also due to these individuals usually have important alterations of lifestyle factors including unhealthy dietary habits or physical activity behaviour [5]. It is therefore reasonable that the positive effects on physical fitness and body composition induced by physical exercise could be partially modulated by changes in vitamin D metabolism. We have recently shown that the PAR, HIIT, and HIT+EMS are effective to improve physical fitness [42] and body composition [43] in our study' cohort. Remarkably, the current findings support the notion that exercise-induced changes in physical fitness are closely related to changes in $1,25(OH)_2D$ which is in line with the previous rationale, while no association was obtained between changes in $1,25(OH)_2D$ and changes in body composition. Further studies are needed to well-understand these associations.

Limitations

The present study had several of limitations. We only included healthy sedentary adults (45-65 years old), and hence, we do not know whether these results can be extended to younger, older, and/or physically active individuals. The sample size of this study was relatively small, so our study may have been underpowered to detect statistical differences in $1,25(OH)_2D$ between the different training modalities, although we observed a robust increment of the $1,25(OH)_2D$ in PAR, HIIT, and HIIT+EMS groups compared with the control group. Further trials involving a greater number of participants are needed to accurately determine training-induced changes when comparing these three exercise methodologies. Finally, we did not measure 25(OH)D levels, which would have allowed us to better understand the role of exercise on vitamin D metabolism.

CONCLUSIONS

In summary, our results show that a 12-week exercise intervention produced an increment of $1,25(OH)_2D$ independently of age, sex, and exercise modality in healthy sedentary adults. Furthermore, we also found a significant positive association between changes in physical fitness and changes in $1,25(OH)_2D$ in our study' cohort. Therefore, we suggest that the link between an exercise intervention and the increase of physical fitness could be mediate changes in vitamin D metabolism.

PERSPECTIVES

The present findings suggest that a concurrent training programs based on physical activity recommendation from the World Health Organization, as well as high intensity interval training programs adding or not whole-body electromyostimulation can be used as a strategy to increase $1,25(OH)_2D$ in middle-aged adults, obtaining slightly better results with the application of a HIIT-EMS program. This conclusion supports the implementation of physical exercise as a strategy not only aiming to reverse the seasonal decrease of $1,25(OH)_2D$ in winter explained by low sunlight exposure (i.e., we performed the baseline assessment in September and the post-intervention test in December), but also for obtaining subsequent increases of this hormone even in these a priori adverse conditions.

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GENERAL DISCUSSION

Under the generic term of vitamin D, a series of different substances are grouped, each of them with specific names. Vitamin D, also called vitamin D3, is cholecalciferol or calciferol, and is synthesized in the skin or ingested through the diet as ergocalciferol or vitamin D2 [1]. Both molecules are doubly hydroxylated in the liver obtaining 25(OH)D (also called calcidiol or calcifediol), and subsequently in the kidney forming 1,25(OH)$_2$D (also called calcitriol), which is the biologically active hormone D [2]. This last hydroxylation is strictly controlled by circulating levels of calcitriol itself, phosphate, and calcium through the parathormone [2]. This regulation involves the synthesis of calcitriol or, alternatively, 24,25-dihydroxy-cholecalciferol which is inactive in human' metabolism [2]. The main function of calcitriol is to increase intestinal absorption and renal retention of calcium, as well as bone mineralization [3]. Calcitriol deficit promotes rickets in children and osteomalacia in adults, among other bone metabolism disturbances [4]. In fact, the majority of human' tissues express calcitriol receptors (i.e., VDRs) which are described as transcription factors of a series of genes involved in the management of calcium [5]. Considering that this is an important intracellular messenger, it is understood that calcitriol action has a pleiotropic nature affecting multiple physiological functions. Indeed, the absence of adequate levels of calcitriol is an important cause of several dysfunctionalities and increased susceptibility to develop various metabolic diseases, some of them associated with the aging process [6]. Similarly, optimal circulating levels of hormone D ensure an optimal functioning of multiple systems, organs and tissues [6]. Concretely, optimal levels of vitamin D even seem to have an anti-aging effect (plasma levels of calcidiol ≥30 ng/ml which can be achieved with a dose of 4,000 IU (100 mcg) of vitamin D) [6]. However, in certain cases and to achieve specific benefits, calcidiol doses and levels are needed to be higher than 80 ng ml [6]. Calcitriol can also be measured in circulation but, given its lower concentration and short half-life, few data are available in the scientific literature. Studies are therefore needed to analyze these levels in a healthy adult population and to describe whether these values are related to other aging biomarkers or if they can be modified with anti-aging interventions such as physical exercise.

We investigated the association between 1,25(OH)$_2$D with body composition (**Study 1**) [7], sedentary behaviour, physical activity levels, and physical fitness (**Study 2**) [8], cardiometabolic risk (**Study 3**) [9] and S-Klotho protein (**Study 4**) [10] in middle-aged sedentary adults. Furthermore, previous studies have described that an exercise intervention induces important benefits on human health [11]. However, the effects of physical exercise on 1,25(OH)$_2$D have not deeply studied. Moreover, there is no study comparing the influence of different exercise interventions on 1,25(OH)$_2$D. We reviewed the available literature in order to determine the effect of exercise on 1,25(OH)$_2$D and, subsequently, we designed a randomized controlled trial aiming to determine

the effects of different exercise training interventions on $1,25(OH)_2D$ (**Study 5**) [12] in middle-aged sedentary adults.

$1,25(OH)_2D$ AND BODY COMPOSITION

Disturbances in body composition including a decrease of lean body mass and/or BMD, or an increment of fat body mass are powerful predictors of morbidity and mortality risk as well as overall quality of life [13]. Vitamin D is a fat-soluble vitamin essential for calcium maintenance homeostasis, bone health, and preventing fractures and falls [14]. Globally, vitamin D deficiency has been recognized as a health problem that probably affects not only musculoskeletal health but also a wide range of several age-related chronic diseases [14]. The relationship between $1,25(OH)_2D$ and body composition parameters has been challenging to establish. Consequently, epidemiological data are scarce, and a review of the scientific literature found no large studies examining the relationship between $1,25(OH)_2D$ with body composition outcomes. There is some evidence of the existence of an inverse association of $1,25(OH)_2D$ with BMI and fat body mass [15]. Other studies found a significant association between low $1,25(OH)_2D$ and low lean body mass [16]. Moreover, previous cross-sectional studies have reported an inverse association between $1,25(OH)_2D$ with BMD [17]. Understanding whether $1,25(OH)_2D$ are associated with body composition parameters in a cohort of middle-aged healthy individuals is of clinical interest since, as previously established, the interventions to reverse or delay body composition related diseases are preferable when individuals are still healthy and relatively young.

The results of the current International Doctoral Thesis show that $1,25(OH)_2D$ is negatively associated with BMI, LMI, and BMD independently of age and sex, while no significant relationship was obtained between $1,25(OH)_2D$ with FMI in middle-aged sedentary adults (**Study 1**) [7]. Intervention studies are therefore needed to understand whether changes in body composition status are associated with changes in $1,25(OH)_2D$ in this age-population.

SEDENTARY BEHAVIOUR, PHYSICAL ACTIVITY LEVELS AND PHYSICAL FITNESS AND $1,25(OH)_2D$

Physical inactivity is recognized as an important public health problem [18] being independently associated with poor life quality, lower life expectancy, higher incidence of chronic diseases, and an increased risk of all-cause mortality [19–22]. Previous studies have reported that cardiorespiratory fitness and muscular strength are also important predictors of cardiometabolic risk [23], all-cause mortality [24–26], and life expectancy [27,28]. It is therefore of scientific interest to identify factors that

explain the relationships of sedentary time, PA levels, and physical fitness with general health during adulthood to well-understand the physio-pathological mechanisms that occur during the aging process.

It has been shown that higher PA levels are associated with higher vitamin D levels in different populations [29–34], but there are controversial findings regarding the relationship between sedentary behaviour and vitamin D levels [29,32,35]. Moreover, limited studies have assessed the main active metabolite of vitamin D (i.e., 1,25(OH)$_2$D). Only a few recent studies have examined the associations of PA levels and sedentary behaviour with 1,25(OH)$_2$D in elderly men [36] and patients with colorectal adenoma [32] obtaining contradictory results. Furthermore, it has been reported a modest positive association between 25(OH)D and cardiorespiratory fitness [37]. However, to date, there are no studies exploring the relationship between cardiorespiratory fitness and 1,25(OH)$_2$D while controversial evidence has been observed regarding the association of muscular strength and 25(OH) [38–43]. The results of the present Doctoral Thesis suggest that objectively measured sedentary time and PA levels are not associated with 1,25(OH)$_2$D in middle-aged sedentary adults. Similarly, no association was found between physical fitness and 1,25(OH)$_2$D in middle-aged sedentary adults (**Study 2**) [8]. These findings support the idea that although vitamin D could be related to the above-mentioned health markers -given their importance on physiological homeostasis-, it seems that 1,25(OH)$_2$D plasma levels are not related to either sedentary behaviour, PA levels, and physical fitness in healthy individuals with relatively adequate values of 1,25(OH)$_2$D.

1,25(OH)$_2$D AND CARDIOMETABOLIC RISK

The incidence of chronic cardiometabolic disorders has dramatically increased during the last decades representing the leading cause of morbidity and mortality in the developed world [44–46]. In this context, the identification of potential biomarkers capable of detecting the risk and progression of cardiometabolic disease is a major goal of clinical medicine for promoting general health [47,48]. Vitamin D deficiency is highly prevalent in different populations across the world [5,49,50]. Indeed, a growing body of scientific works has investigated the physio-pathological mechanisms behind cardiovascular disease, suggesting that vitamin D deficiency could play a key role in its development [51–54].

Previous studies have examined whether 25(OH)D deficiency is associated with a higher risk of suffering cardiovascular disease obtaining controversial findings [52,55–59]. However, considerably less attention has been paid to the relationship between 1,25(OH)$_2$D and

cardiometabolic risk factors. Given that identifying new potential biomarkers to detect cardiometabolic alterations in still healthy subjects potentially allows to apply preventive strategies and that they are preferable to the treatment of cardiometabolic diseases already established, it seems of scientific interest to determine whether 1,25(OH)$_2$D levels are associated with cardiometabolic risk factors in individuals free of chronic diseases [60,61]. The results of this Doctoral Thesis show that 1,25(OH)$_2$D plasma levels are associated with neither the cardiometabolic risk factors nor insulin resistance in healthy sedentary adults (**Study 3**) [9]. However, we observed that higher 1,25(OH)$_2$D plasma levels were consistently associated with low central adiposity in our study cohort. These findings support the idea that although 1,25(OH)$_2$D has been proposed as a key factor affecting cardiometabolic health in patients with chronic diseases [62,63], it seems that 1,25(OH)$_2$D plasma levels are not related to cardiometabolic risk factors in healthy individuals with adequate values of these physiological parameters.

1,25(OH)$_2$D AND S-KLOTHO

Recent studies have established the importance of vitamin D status on human' health during the ageing process beyond its role on specific physiological mechanisms in different organs and systems [5]. Vitamin D alterations have been consistently linked to greater incidence and prevalence of several age-related chronic diseases, impaired physical function, and mortality in different populations [64–66]. Previous studies have reported important anti-aging functions of 1,25(OH)$_2$D specifically in bone mineral metabolism [36], oxidative stress [67], neurological functions [68], energy metabolism [69] and cardiovascular health [4].

In humans, S-Klotho has been proposed as an accurate indicator of renal α-Klotho protein expression [70], an anti-aging gene, which extends life expectancy when is over-expressed, and accelerates aging-like phenotypes when is under-expressed [71]. S-Klotho is therefore considered a powerful anti-aging biomarker in healthy humans [72], which could maintain a reciprocal interaction with 1,25(OH)$_2$D [73]. Murine studies [74–77] have shown that 1,25(OH)$_2$D stimulates the expression of the α-Klotho gene and FGF23 while increased levels of S-Klotho and FGF23 inhibit 1-α-hydroxylase leading to the lowering 1,25(OH)$_2$D synthesis and its subsequent degradation [73]. Understanding the interaction between these two anti-aging biomarkers in middle-aged adults is of clinical interest since studying aging in the elderly population suffers the limitation that the majority of individuals already have some form of aging-related disease [47,60].

The findings of the present Doctoral thesis results suggest that 1,25(OH)$_2$D is slightly inversely associated with S-Klotho, with this association being increased after adjusting by FMI,

and attenuated after adjusting by age and sex (**Study 4**) [10]. Interestingly, BMD significantly explained the association between these two variables. To the best of our knowledge, this is the first study that elucidates an inverse relationship between $1,25(OH)_2D$ and S-Klotho plasma levels in humans.

ROLE OF EXERCISE ON $1,25(OH)_2D$

In addition to medical treatment, physical exercise has been postulated as an auspicious strategy to counteract both mental and physical chronic disorders [78]. Concretely, Pedersen and Saltin proposed physical exercise as the most important instrument to prevent and treat more than thirty-five chronic diseases (e.g., psychiatric disorders, metabolic pathologies, cardiovascular diseases or cancer among others) [79].

Previous scientific evidence has demonstrated the efficacy of physical exercise to prevent and/or delay the deleterious effects of the aging process on physiological functions [80]. Nevertheless, it remains partially unknown the molecular and biological pathways that explain the exercise-related positive influence on human' health in old age. In this regard, considerably little attention has been focused upon physical exercise as a regulator of vitamin D status. Interestingly, it has been reported that an aerobic training intervention induced a significant increment of $25(OH)D$ in elderly adults [81,82]. However, to the best of our knowledge, there is no study investigating the effects of physical exercise on $1,25(OH)_2D$. From a preventive perspective, it is of scientific and clinical interest to study these metabolites in relatively young individuals free of chronic disorders [83]. Furthermore, no data are yet available concerning whether different exercise training modalities could induce contrasting effects on $1,25(OH)_2D$.

The results of the present Doctoral Thesis were that, compared to a control group, the participants included in the exercise intervention groups benefited from a significant improvement in $1,25(OH)_2D$ independently of the exercise modality (**Study 5**) [12]. Interestingly, while no significant association between changes in body composition and changes in $1,25(OH)_2D$ was observed, a significant positive association was found between changes in physical fitness and changes in $1,25(OH)_2D$. Taking all together, these results suggest that physical exercise is an effective strategy to increase $1,25(OH)_2D$ in healthy sedentary adults, and that these training effects may partially explain changes in physical fitness.

GENERAL LIMITATIONS

The findings of the present International Doctoral Thesis should be considered with caution due to a number of limitations. Specific limitations of each study are presented in the discussion section of each study and an overall view of the main limitations is presented here:

- Four out of five studies contained in the Doctoral Thesis had cross-sectional designs, and thus, it is not possible to establish a causal relationship in the associations observed between $1,25(OH)_2D$ and the anti-aging factors analyzed. Future exploratory and confirmatory trials are needed to verify our findings.

- All the studies were carried out in a cohort of middle-aged sedentary adults (45 to 65 years old). Therefore, these data should not be extrapolated to other populations with different biological characteristics. Therefore, it is mandatory to replicate the present studies on different populations (i.e., younger, older, physically active, and/or individuals with different ethnicity and health status).

- Specific information on sun exposure or time spent outdoors was not recorded, and although objectively measured sedentary time and physical activity were used as covariates in the statistical analyses, this does not fully reflect sun exposure. Carefully collected data on sun exposure and sun protective behaviors during physical activity and/or sedentary time are necessary in future studies.

- All studies of this Doctoral Thesis included data of $1,25(OH)_2D$. However, no other circulating vitamin D measures such as $25(OH)D$ were assessed. Further studies should measure these outcomes to well-understand the current findings.

- All studies of this Doctoral Thesis included $1,25(OH)_2D$, S-Klotho, and several cardiometabolic risk factors status data obtained by a single sample assay.

- The sample size of FIT-AGEING study was relatively small, so our data should be interpreted with caution. Moreover, due to the sample size, the Study 5 may have been underpowered to detect statistical differences in $1,25(OH)_2D$ between the different training modalities, although we observed a robust increment of the $1,25(OH)_2D$ in PAR, HIIT, and HIIT+EMS groups compared with the control group. Further trials involving a greater number of participants are needed to accurately determine training-induced changes when comparing these three exercise methodologies.

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CONCLUDING REMARKS AND FUTURE PERSPECTIVES

GENERAL CONCLUSION

Collectively, the results from the present International Doctoral Thesis enhance our understanding of the relationship of 1,25(OH)$_2$D with several anti-aging factors such as body composition, physical activity levels, physical fitness, cardiometabolic health, and S-Klotho protein in middle-aged sedentary adults. Moreover, it provides novel information regarding the role of different exercise training programs on 1,25(OH)$_2$D, as well as its potential relationship with exercise-induced changes in body composition and physical fitness. We initially expected positive associations between 1,25(OH)$_2$D levels and the different anti-aging markers as it has been consistently reported for 25(OH)D. It is mportant to clarify this issue given the limited number of studies regarding 1,25(OH)$_2$D and the conflictive results that these studies report. In contrast to our initial hypothesis what we have found, and we want to highlight, is that 1,25(OH)$_2$D was negatively associated with body mass index, lean mass index, and bone mineral density, as well as that objectively measured sedentary time, physical activity levels, and physical fitness did not seem to be related to 1,25(OH)$_2$D. Subsequently, no association was found between 1,25(OH)$_2$D and cardiometabolic risk factors or insulin resistance, although an inverse association between 1,25(OH)$_2$D and central adiposity was observed. Furthermore, we evidenced that 1,25(OH)$_2$D was negatively associated with S-Klotho plasma levels, which was partially mediated by bone mineral density. Finally, we found that a 12-weeks exercise intervention significantly increased 1,25(OH)$_2$D independently of the exercise modality, and we observed a significant positive association between exercise-induced changes in physical fitness and changes in 1,25(OH)$_2$D in this study cohort.

SPECIFIC CONCLUSIONS

The specific conclusions reached in the studies included in this International Doctoral Thesis are detailed as follow:

SECTION 1: 1,25-dihydroxyvitamin D and ageing markers: body composition, physical activity levels, physical fitness, cardiometabolic health, and S-Klotho protein

- **Study 1.** Higher levels of 1,25(OH)$_2$D are associated with lower body mass index, lean mass index, and bone mineral density independently of age and sex, while 1,25(OH)$_2$D do not to be related to fat mass index in middle-aged sedentary adults.

- **Study 2.** Objectively measured sedentary time, physical activity levels, and physical fitness are not associated with 1,25(OH)$_2$D in middle-aged sedentary adults.

- **Study 3.** 1,25(OH)$_2$D is associated with neither cardiometabolic risk factors nor insulin resistance in healthy sedentary adults, independently of potential confounders such as age, sex, body mass index, and/or fat mass index. However, an inverse association of 1,25(OH)$_2$D with central adiposity was observed in this study cohort.

- **Study 4.** 1,25(OH)$_2$D is slightly inversely associated with S-Klotho in middle-aged sedentary adults, with this association being increased after adjusting by fat mass index, and attenuated after adjusting by age and sex. Moreover, bone mineral density significantly explained the association between these two variables.

SECTION 2: Role of exercise on 1,25-dihydroxyvitamin D

- **Study 5.** A 12-week exercise intervention produced an increment of 1,25(OH)$_2$D independently of age, sex, and exercise modality in healthy sedentary adults. Moreover, while no significant association between exercise-induced changes in body composition and changes in 1,25(OH)$_2$D was observed, a significant positive association was found between changes in physical fitness and changes in 1,25(OH)$_2$D.

FUTURE PERSPECTIVES

- Future randomized controlled trials with simultaneous measurement of calcitriol and calcidiol, and applying similar physical exercise protocols in healthy individuals with different ages and in patients with metabolic disorders (i.e., metabolic syndrome) are necessary to further investigate if these results apply to people with different biological characteristics.

- Considering that 1,25(OH)$_2$D could be modulated by nutrition, future studies are needed to investigate if novel training methodologies and nutritional interventions such as HIIT+EMS combined with time-restricted eating provides extra benefits on human health during the ageing process.

- The results of the present Doctoral Thesis suggest that HIIT+EMS provides additive benefits in 1,25(OH)$_2$D status. However, future studies should test whether a PAR intervention plus WB-EMS induces similar or even greater effects 1,25(OH)$_2$D compared with a HIIT+EMS intervention.

- Further studies should determine not only $1,25(OH)_2D$, but also $25(OH)D$ to better understand the role of physical exercise on vitamin D metabolism. In addition, it is necessary to investigate whether $1,25(OH)_2D$ and $25(OH)D$ changes induced by an exercise intervention could be mediated by changes in other health-related outcomes.

- Finally, it is worth investigating the possible divergent role of an active hormone $(1,25(OH)_2D)$ and its inactive precursor $(25(OH)D)$. In this regard, good availability of the hormone, in the form of its inactive precursor $(25(OH)D)$, is convenient in order to face possible stressful circumstances (i.e. exercise), under which the precursor is activated $(1,25(OH)_2D)$ and its precursor decline. This hypothesis may explain this divergence and is worth to be investigated.

ANNEXES

BOOK CHAPTERS AND PAPERS DERIVED FROM THE DOCTORAL THESIS

PUBLISHED & INCLUDED IN THIS DOCTORAL THESIS

1   **De la O Puerta A**, Amaro Gahete FJ y Castillo Garzón MJ. Nutrición deportiva: desde la fisiología a la práctica. Capítulo 34. Ejercicio físico y nutrición: papel en la salud y efecto antienvejecimiento. Panamericana, 2020. ISBN: 9788491106036.

2   **De-la-O A**, Jurado-Fasoli L, Castillo MJ, Gracia-Marco L, Gutiérrez Á, Amaro-Gahete FJ. Relationship between 1,25-Dihydroxyvitamin D and Body Composition in Middle-Aged Sedentary Adults: The FIT-AGEING Study. *Nutrients* 2019; **11**: 2567.

3   **De-la-O A**, Jurado-Fasoli L, Castillo MJ, Gutiérrez Á, Amaro-Gahete FJ. 1,25-Dihydroxyvitamin D and S-Klotho plasma levels: The relationship between two renal anti-aging biomarkers mediated by bone mineral density in middle-aged sedentary adults. *Rejuvenation Res* 2021. In press.

SUBMITTED & INCLUDED IN THIS DOCTORAL THESIS

1   **De-la-O A**, Castillo-Gualda P, Amaro-Gahete FJ, Castillo Garzón MJ. Vitamin D/ Calcidiol/ Calcitriol: Role in ageing and anti-aging. *Under review*

2   **De-la-O A**, Jurado-Fasoli L, Castillo MJ, Gutiérrez A, Amaro-Gahete FJ. Relationship of sedentary time, physical activity and fitness with 1,25-Dihydroxyvitamin D in middle-aged sedentary adults: The FIT-AGEING study. *Under review*

3   **De-la-O A**, Jurado-Fasoli L, Castillo MJ, Gutiérrez A, Amaro-Gahete FJ. 1,25-Dihydroxyvitamin D and cardiometabolic risk in healthy sedentary adults: The FIT-AGEING Study. *Under review*

4   **De-la-O A**, Jurado-Fasoli L, Castillo MJ, Gutiérrez A, Amaro-Gahete FJ. 1,25-Dihydroxyvitamin D is increased in response to different exercise training interventions in healthy sedentary adults: the FIT-AGEING randomized controlled trial. *Under review*

SHORT CURRICULUM VITAE

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4. Education

2009	Degree in Teaching of Physical Education (Grade 9.1/10), University of Granada, Spain.
2015	Bachelor´s degree in Physical Activity and Sports Sciences (Grade 8.9/10), University of Granada, Spain.
2015	Master´s degree in Researching in Physical Activity and Sport (Grade: 9.3/10), University of Granada, Spain.
2016 – 2021	PhD Student in Biomedicine, University of Granada, Spain.

5. Previous positions

2014	Research Initiation Fellow. Department of Physiology, Faculty of Medicine, University of Granada, Granada, Spain.
2015	Research Initiation Fellow for Master´s Student. Department of Physiology, Faculty of Medicine, University of Granada, Granada, Spain.
2015 – 2021	Predoctoral FPU Research Fellow. Department of Physiology, Faculty of Medicine, University of Granada, Granada, Spain.

6. Supervision

2017 – 2018	Professional tutor of external practices. Degree in Physical Activity and Sport Sciences, University of Granada, Spain.
2018 – 2019	Professional tutor of external practices. Degree in Physical Activity and Sport Sciences, University of Granada, Spain.

7. Research projects

2015	C-HIPPER Project: Climbing High Performance International Project.
2015 – present	BEER-HIIT Project: Beer or Ethanol Effects on the Response to High Intensity Interval Training: A Controlled Study in Healthy Individuals.
2015 – present	FIT-AGEING Project: Exercise training as S-Klotho protein stimulator in sedentary healthy adults.

8. Courses attended (Transferable skills)

29/01/2016 – 29/01/2016	Course: European projects on the H2020 horizon.
20/04/2016 – 20/04/2016	PhD course: How to get published in international journal.
14/03/2017 – 16/03/2017	PhD course: Search and information management tools for research development.
06/04/2017 – 21/05/2017	Course: High-intensity aerobic interval training (HIT): from physiology to practice.
22/04/2017 – 22/04/2017	Course: Sport nutrition.
07/06/2017 – 07/06/2017	Course: Physical exercise and bone health.
23/01/2018 – 24/01/2018	PhD course: Statistical analysis of randomized clinical trials.
03/04/2018 – 08/06/2018	PhD Course: How to write and publish a scientific article. An applied approach. 9th edition.
15/10/2018 – 19/10/2018	PhD course: Graphic design focused on the scientific field.
20/12/2018 – 20/12/2018	PhD course: New visual ways to communicate clinical and health psychology.
15/01/2019 – 24/01/2019	PhD course: Statistical techniques applied in the field of nutrition and health.
28/01/2019 – 30/01/2019	PhD course: Evidence based medicine: research publication workshop.
06/02/2019 – 06/02/2019	Course: Turnitin for Students.
07/02/2019 – 08/02/2019	PhD course: Systematic review.

9. Publications list

1. **De-la-O A,** Jurado-Fasoli L, Castillo MJ, Gutiérrez Á, Amaro-Gahete FJ. 1,25-Dihydroxyvitamin D and S-Klotho plasma levels: The relationship between two renal anti-ageing biomarkers mediated by bone mineral density in middle-aged sedentary adults. *Rejuvenation Res* 2021. In press.

2. Jurado-Fasoli L, **De-la-O A**, Molina-Hidalgo C, Migueles JH, Castillo MJ, Amaro-Gahete FJ. Exercise training improves sleep quality: a randomized controlled trial. *Eur J Clin Invest* 2020; **50**: e13202.

3. Jurado-Fasoli L, Amaro-Gahete FJ, **De-la-O A**, Castillo MJ. Impact of different exercise training modalities on energy and nutrient intake and food consumption in sedentary middle-aged adults: a randomised controlled trial. *J Hum Nutr Diet* 2020; **33**: 86–97.

4. Molina-Hidalgo C, **De-la-O A**, Dote-Montero M, Amaro-Gahete FJ, Castillo MJ. Influence of daily beer or ethanol consumption on physical fitness in response to a high-intensity interval training program. The BEER-HIIT study. *J Int Soc Sports Nutr* 2020; **17**: 1–13.

5. Amaro-Gahete FJ, **De-la-O A**, Jurado-Fasoli L, Sanchez-Delgado G, Ruiz JR, Castillo MJ. Metabolic rate in sedentary adults, following different exercise training interventions: The FIT-AGEING randomized controlled trial. *Clin Nutr* 2020; **39**: 3230–3240.

6. Navarro-Lomas G, **De-la-O A**, Jurado-Fasoli L, Castillo MJ, Femia P, Amaro-Gahete FJ. Assessment of autonomous nerve system through non-linear heart rate variability outcomes in sedentary healthy adults. *PeerJ* 2020; **8**: e10178.

7. Dote-Montero M, **De-la-O, A**, Castillo MJ, Amaro-Gahete FJ. Predictors of sexual desire and sexual function in sedentary middle-aged adults: the role of lean mass index and S-Klotho plasma levels. The FIT-AGEING study. *J Sex Med* 2020; **17**: 665–677.

8. Iguacel I, Börnhorst C, Michels N, Breidenassel C, Dallongeville J, González-Gross M *et al.* Socioeconomically disadvantaged groups and metabolic syndrome in European adolescents: The HELENA study. *J Adolesc Heal* 2020; **68**: 146–154

9. **De la O Puerta A**, Amaro Gahete FJ y Castillo Garzón MJ. Nutrición deportiva: desde la Fisiología a la Práctica. Capítulo 34. Ejercicio físico y nutrición: efectos para la salud y efecto anti-envejecimiento. Panamericana, 2020. ISBN: 9788491106036.

10. Jurado-Fasoli L, Amaro-Gahete FJ, **De-la-O A**, Martinez-Tellez B, Ruiz JR, Gutierrez A *et al.* Adherence to the Mediterranean diet, dietary factors, and S-Klotho plasma levels in sedentary middle-aged adults. *Exp Gerontol* 2019; **119**: 25–32.

11. Amaro-Gahete FJ, Sanchez-Delgado G, Jurado-Fasoli L, **De-la-O A**, Castillo MJ, Helge JW *et al.* Assessment of maximal fat oxidation during exercise: A systematic review. *Scand J Med Sci Sports* 2019; **29**: 910–921.

12. Amaro-Gahete FJ, **De-la-O A**, Jurado-Fasoli LJ, R. Ruiz J, Castillo MJ. Association of basal metabolic rate and fuel oxidation in basal conditions and during exercise, with plasma S-klotho: the FIT-AGEING study. *Aging (Albany NY)* 2019; **11**: 5319.

13. Amaro-Gahete FJ, **De-la-O A**, Jurado-Fasoli L, Gutiérrez Á, Ruiz JR, Castillo MJ. Association of physical activity and fitness with S-Klotho plasma levels in middle-aged sedentary adults: The FIT-AGEING study. *Maturitas* 2019; **123**: 25–31.

14. Molina-Hidalgo C [φ], **De-la-O A** [φ], Jurado-Fasoli L, Amaro-Gahete FJ, Castillo MJ. Beer or ethanol effects on the body composition response to high-intensity interval training. The BEER-HIIT Study. *Nutrients* 2019; **11**: 909.

15. Amaro-Gahete FJ, **De-la-O A**, Jurado-Fasoli L, Espuch-Oliver A, de Haro T, Gutiérrez Á *et al.* Body composition and S-Klotho plasma levels in middle-aged adults: A cross-sectional study. *Rejuvenation Res* 2019; **22**: 478–483.

16. Amaro-Gahete FJ, **De-la-O A,** Jurado-Fasoli L, Dote-Montero M, Gutierrez A, Ruiz JR *et al.* Changes in physical fitness after 12 weeks of structured concurrent exercise training, high intensity interval training, or whole-body electromyostimulation training in sedentary middle-aged adults: A randomized controlled trial. *Front Physiol* 2019; **10**: 451.

17. Jurado-Fasoli L, **De-la-O A**, Castillo MJ, Amaro-Gahete FJ. Dietary differences between metabolically healthy overweight-obese and metabolically unhealthy overweight-obese adults. *Br J Nutr* 2019; **122**: 1113–1119.

18. Giménez-Legarre N, Santaliestra-Pasías AM, Beghin L, Dallongeville J, **De-la-O A**, Gilbert C *et al.* Dietary patterns and their relationship with the perceptions of healthy eating in European adolescents: the HELENA study. *J Am Coll Nutr* 2019; **38**: 703–713.

19. Amaro-Gahete FJ, Jurado-Fasoli L, Triviño AR, Sanchez-Delgado G, **De-la-O A**, Helge JW *et al.* Diurnal Variation of Maximal Fat-Oxidation Rate in Trained Male Athletes. *Int J Sports Physiol Perform* 2019; **14**: 1140–1146.

20.   Amaro-Gahete FJ, **De-la-O A**, Jurado-Fasoli L, Ruiz JR, Castillo MJ, Gutiérrez Á. Effects of different exercise training programs on body composition: A randomized control trial. *Scand J Med Sci Sports* 2019; **29**: 968–979.

21.   Amaro-Gahete FJ, **De-la-O A**, Jurado-Fasoli L, Martinez-Tellez B, R Ruiz J, Castillo MJ. Exercise training as a treatment for cardiometabolic risk in sedentary adults: are physical activity guidelines the best way to improve cardiometabolic health? The FIT-AGEING randomized controlled trial. *J Clin Med* 2019; **8**: 2097.

22.   Amaro-Gahete FJ, **De-la-O A**, Jurado-Fasoli L, Espuch-Oliver A, de Haro T, Gutierrez A *et al.* Exercise training increases the S-Klotho plasma levels in sedentary middle-aged adults: A randomised controlled trial. The FIT-AGEING study. *J Sports Sci* 2019; **37**: 2175–2183.

23.   **De-la-O A**, Jurado-Fasoli L, Castillo MJ, Gracia-Marco L, Gutierrez Á, Amaro-Gahete FJ. Relationship between 1, 25-Dihydroxyvitamin D and Body Composition in Middle-Aged Sedentary Adults: The FIT-AGEING Study. *Nutrients* 2019; **11**: 2567.

24.   Dote-Montero M, Amaro-Gahete FJ, **De-la-O A**, Jurado-Fasoli L, Gutierrez A, Castillo MJ. Study of the association of DHEAS, testosterone and cortisol with S-Klotho plasma levels in healthy sedentary middle-aged adults. *Exp Gerontol* 2019; **121**: 55–61.

25.   Amaro-Gahete F, Jurado-Fasoli L, **De-la-O A**, Gutierrez Á, Castillo M, Ruiz J. Accuracy and Validity of Resting Energy Expenditure Predictive Equations in Middle-Aged Adults. *Nutrients* 2018; **10**: 1635.

26.   Jurado-Fasoli L, Amaro-Gahete FJ, **De-la-O A**, Gutierrez A, Castillo MJ. Alcohol consumption and S-Klotho plasma levels in sedentary healthy middle-aged adults: A cross sectional study. *Drug Alcohol Depend* 2018; **194**: 107–111.

27.   Jurado-Fasoli L, Amaro-Gahete FJ, **De-la-O A**, Dote-Montero M, Gutiérrez Á, Castillo MJ. Association between sleep quality and body composition in sedentary middle-aged adults. *Medicina (B Aires)* 2018; **54**: 91.

28.   Arouca A, Michels N, Moreno LA, González-Gil EM, Marcos A, Gómez S *et al.* Associations between a Mediterranean diet pattern and inflammatory biomarkers in European adolescents. *Eur J Nutr* 2018; **57**: 1747–1760.

29.   Iglesia I, Huybrechts I, Mouratidou T, Santabárbara J, Fernández-Alvira JM, Santaliestra-Pasías AM *et al.* Do dietary patterns determine levels of vitamin B6, folate, and vitamin B12 intake and corresponding biomarkers in European adolescents? The Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) study. *Nutrition* 2018; **50**: 8–17.

30.   Amaro-Gahete FJ, **De-la-O A**, Jurado-Fasoli L, Espuch-Oliver A, Robles-Gonzalez L, Navarro-Lomas G *et al.* Exercise training as S-Klotho protein stimulator in sedentary healthy adults: Rationale, design, and methodology. *Contemp Clin Trials Commun* 2018; **11**: 10–19.

31.   Amaro-Gahete FJ, **De-la-O A**, Sanchez-Delgado G, Robles-Gonzalez L, Jurado-Fasoli L, Ruiz JR *et al.* Functional exercise training and undulating periodization enhances the effect of whole-body electromyostimulation training on running performance. *Front Physiol* 2018; **9**: 720.

32.   Fryer SM, Giles D, Palomino IG, **de la O Puerta A**, España-Romero V. Hemodynamic and cardiorespiratory predictors of sport rock climbing performance. *J Strength Cond Res* 2018; **32**: 3534–3541.

33.   Amaro-Gahete FJ, **De-la-O A**, Jurado-Fasoli L, Ruiz JR, Castillo MJ, Gutiérrez Á. Role of exercise on S-Klotho protein regulation: A systematic review. *Curr Aging Sci* 2018; **11**: 100–107.

34.   Amaro-Gahete FJ, **De-la-O A**, Sanchez-Delgado G, Robles-Gonzalez L, Jurado-Fasoli L, Ruiz JR *et al.* Whole-body electromyostimulation improves performance-related parameters in runners. *Front Physiol* 2018; **9**: 1576.

35.   Julian C, González-Gross M, Breidenassel C, Mouratidou T, Vicente-Rodriguez G, Gracia-Marco L *et al.* 25-hydroxyvitamin D is differentially associated with calcium intakes of Northern, Central, and Southern European adolescents: Results from the HELENA study. *Nutrition* 2017; **36**: 22–25.

36.   Iglesia I, González-Gross M, **de la O Puerta A**, Mouratidou T, De-Miguel-Etayo P, Gottrand F *et al.* Associations between insulin resistance and three B-vitamins in European adolescents: the HELENA study. *Nutr Hosp* 2017; **34**: 568–577.

37.   Esteban-Cornejo I, Cadenas-Sanchez C, Vanhelst J, Michels N, Lambrinou C-P, Gonzalez-Gross M *et al.* Attention capacity in European adolescents: role of different health-related factors. The HELENA study. *Eur J Pediatr* 2017; **176**: 1433–1437.

38.   Amaro-Gahete FJ, **de la O A**, Jurado-Fasoli L, Ruiz JR, Gutierrez A. Could superimposed electromyostimulation be an effective training to improve aerobic and anaerobic capacity? Methodological considerations for its development. *Eur. J. Appl. Physiol.* 2017; **117**: 1513–1515.

39.   Julian C, Mouratidou T, Vicente-Rodriguez G, Gracia-Marco L, Valtueña J, González-Gross M *et al.* Dietary sources and sociodemographic and lifestyle factors affecting vitamin D and calcium intakes in European adolescents: the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) Study. *Public Health Nutr* 2017; **20**: 1593–1601.

40.   Giles D, Romero VE, Garrido I, **de la O Puerta A,** Stone K, Fryer S. Differences in oxygenation kinetics between the dominant and nondominant flexor digitorum profundus in rock climbers. *Int J Sports Physiol Perform* 2017; **12**: 137–139.

41.   Amaro Gahete FJ, **De-la-O A,** Jurado-Fasoli L, Castillo MJ, Gutiérrez Sáinz Á. Fitness assessment as an anti-aging marker: a narrative review. *J Gerontol Geriatr Res* 2017; **6**: 455.

42.   González-Gil EM, Santabarbara J, Ruiz JR, Bel-Serrat S, Huybrechts I, Pedrero-Chamizo R *et al.* Ideal cardiovascular health and inflammation in European adolescents: The HELENA study. *Nutr Metab Cardiovasc Dis* 2017; **27**: 447–455.

43.   Amaro-Gahete FJ, **De-la-O A**, Robles-González L, Castillo MJ, Gutiérrez Á. Impact of two whole-body electromyostimulation training modalities on body composition in recreational runners during endurance training cessation. *RICYDE Rev Int Ciencias del Deport* 2017; **14**: 205–218.

44.   **de la O Puerta A**, Manzano SS, Sáinz AG. *Efecto de la fatiga sobre el tiempo de reacción en dominante y no dominante en pádel: Estudio piloto.* Innovación e investigación en pádel. Wanceulen Editorial Deportiva, 2016

45.   **de la O A**, Amaro F, Roero C, Gutiérrez A. Influencia de tres tipos diferentes de entrenamiento (Electroestimulación global, High Intensity Interval Training (HIIT) y Aerobio convencional) sobre el metabolismo basal post esfuerzo. *Rev Andaluza Med del Deport* 2015; **8**: 27.

ϕ Equally contributed

10. Papers under review

1.   **De-la-O A**, Castillo-Gualda P, Amaro-Gahete FJ, Castillo Garzón MJ. Vitamin D/ Calcidiol/ Calcitriol: Role in ageing and anti-ageing.

2.   **De-la-O A,** Jurado-Fasoli L, Castillo MJ, Gutiérrez A, Amaro-Gahete FJ. Relationship of sedentary time, physical activity and fitness with 1,25-Dihydroxyvitamin D in middle-aged sedentary adults: The FIT-AGEING study.

3.   **De-la-O A**, Jurado-Fasoli L, Castillo MJ, Gutiérrez A, Amaro-Gahete FJ. 1,25-Dihydroxyvitamin D and cardiometabolic risk in healthy sedentary adults: The FIT-AGEING Study.

4.   **De-la-O A**, Jurado-Fasoli L, Castillo MJ, Gutiérrez A, Amaro-Gahete FJ. 1,25-Dihydroxyvitamin D is increased in response to different exercise training interventions in healthy sedentary adults: the FIT-AGEING randomized controlled trial.

5.   **De-la-O A**$^{\phi}$, Jurado-Fasoli L$^{\phi}$, Gracia-Marco L, Henriksson P, Gutiérrez A, Amaro-Gahete FJ. Association of energy and macronutrients intake with S-Klotho plasma levels in middle-aged sedentary adults: a cross-sectional study.

$^{\phi}$ Equally contributed

11. Other merits

2014 – present	Co-author of more than 20 congress communications (including national and international conferences).
2017 – 2020	Lecturer in the degree of Physiotherapy. University of Granada.
2017 – 2020	Lecturer in the degree of Medicine. University of Granada.
2019 – 2020	Lecturer in the degree of Occupational Therapy. University of Granada.