International Doctoral Thesis / Tesis Doctoral Internacional

Physical exercise as a modulator of the anti-ageing Klotho protein: health-related cardiometabolic implications. The FIT-AGEING study

El ejercicio físico como modulador de la proteína antienvejecimiento Klotho: implicaciones cardiometabólicas relacionadas con la salud. Estudio FIT-AGEING

PROGRAMA DE DOCTORADO EN BIOMEDICINA
DEPARTAMENTO DE FISIOLOGÍA
FACULTAD DE MEDICINA
UNIVERSIDAD DE GRANADA
Francisco J. Amaro Gahete
2019

Editor: Universidad de Granada. Tesis Doctorales
Autor: Francisco J. Amaro Gahete
ISBN: 978-84-1306-314-0
URI: http://hdl.handle.net/10481/57263

El doctorando D. Francisco J. Amaro Gahete ha realizado la presente Tesis Doctoral como beneficiario de una beca-contrato con cargo al programa de Formación de Profesorado Universitario (FPU14/04172) del Ministerio de Educación, Cultura y Deporte, por resolución de 20 de agosto de 2015, de la Secretaría de Estado de Educación, Formación Profesional y Universidades (BOE-A-2015- 9456).

A mi padre, madre y hermano por enseñarme a través del cariño, el amor y la perseverancia el verdadero valor las cosas

A Lidia, por acompañarme y apoyarme incondicionalmente en mi proyecto vital

TABLE OF CONTENTS

RESEARCH PROJECTS AND FUNDING

The present International Doctoral Thesis was mainly performed under the framework of the FIT-AGEING study which did not receive any direct funding from public or private organizations.

ABBREVIATIONS

1RM: One-repetition maximum

ALT: Alanine transaminase

ANCOVA: Analysis of covariance

ANOVA: Analysis of variance

BFox: Basal fat oxidation

BCHox: Basal carbohydrate oxidation

BMC: Bone mineral content

BMD: Bone mineral density

BMI: Body mass index

BMR: Basal metabolic rate

BOCF: baseline observation carried forward imputation

CV: Coefficient of variance

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

Erl1/2: Extracellular signal-regulated protein kinases 1 and 2

Fat_{max}: Intensity that elicit MFO

FFM: Fat free mass

FGF: Fibroblast growth factor

FGFR: Fibroblast growth factor receptor

FM: Fat mass

FMI: Fat mass index

FOXO: Forkhead box protein O

HC: Hip circumference

HDL-C: High-density lipoprotein cholesterol

HIIT: High intensity interval training

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

HOMA: Homeostatic model assessment of insulin resistance index

HRres: Heart rate reserve

IC: Indirect calorimetry

IFNγ: Interferon gamma

IGF-1: Insulin-like growth factor-1

IL: Interleukin

LDL-C: Low-density lipoprotein cholesterol

LM: Lean mass

LMI: Lean mass index

LPA: Light physical activity time

MFO: Maximal fat oxidation during exercise

MVPA: Moderate-vigorous physical activity time

MPA: Moderate physical activity time

NaPi-2: Sodium phosphate co-transporter type-2

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

PCA: Principal-components analysis

PI3K: Phosphatidylinositol 3-kinase

PPAR-γ: Peroxisome proliferator-activated receptor-γ

PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses

QUICKI: Quantitative insulin sensitivity check index

REE: Resting energy expenditure

RER: Respiratory exchange ratio

SGK: Serine/threonine-protein kinase

RIG-1: Retinoic-acid-inducible gene-I

RPE: Rating of perceived exertion

S-Klotho: Shed form of the Klotho protein

SD: Standard deviation

TGF-β: Transforming growth factor beta

TNF-α: Tumor necrosis factor alfa

TRPV: Transient receptor potential cation channels

UCP3: Uncoupling protein 3

VAT: Visceral adipose tissue

VCO_2: Carbon dioxide production

VT2: Ventilatory threshold 2

VO_2: Oxygen uptake

VO_2max: Maximal oxygen uptake

VPA: Vigorous activity time

WB-EMS: Whole-body electromyostimulation training

WC: Waist circumference

WHr: Waist-hip ratio

γ-GT: γ-glutamyl transferase

PAPERS DERIVED FROM THE INTERNATIONAL DOCTORAL THESIS

1 **Amaro Gahete FJ**, De La O A, Jurado Fasoli L, Castillo MJ, Gutierrez A. Fitness Assessment as an Anti-Aging Marker: A Narrative Review. ***J Gerontol Geriatr Res*** 2017; 06 (6): 455

2 **Amaro-Gahete FJ**, de la O A, Jurado-Fasoli L, Ruiz JR, Castillo MJ, Gutierrez A. Role of exercise on S-Klotho protein regulation: a systematic review. ***Curr Aging Sci*** 2018; **11** (2): 100-107**.**

3 **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.

4 **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. In press

5 **Amaro-Gahete FJ**, Sanchez-Delgado G, Alcantara JMA, Martinez-Tellez B, Acosta FM, Helge JW *et al.* Impact of data analysis methods for maximal fat oxidation estimation during exercise in sedentary adults. ***Eur J Sport Sci*** 2019; **28**: 1–10.

6 **Amaro-Gahete FJ**, Sanchez‐Delgado G, Helge JW, Ruiz J. Optimizing maximal fat oxidation assessment by a treadmill-based graded exercise protocol: when should the test end? *Submitted*.

7 **Amaro-Gahete FJ,** Sanchez-Delgado G, Ruiz JR. Commentary: Contextualising Maximal Fat Oxidation During Exercise: Determinants and Normative Values. ***Front Physiol*** 2018; **9**: 1460.

8 **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; **31**: 1–20.

9 **Amaro-Gahete FJ**, 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.

10 **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. In press.

11 **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 sedentary

middle-aged adults: the FIT-AGEING study. ***Maturitas*** 2019; **123**: 25-31.

12 **Amaro‐Gahete FJ**, De-la-O A, Jurado-Fasoli, Ruiz J, Castillo M. Association of basal metabolic rate and fuel oxidation in basal conditions and during exercise, with plasma S-Klotho. *Submitted*.

13 **Amaro‐Gahete FJ,** Jurado-Fasoli L, De-la-O A, Ruiz J, Castillo M. Relationship of S-Klotho and cardiometabolic risk in sedentary middle-aged adults: the FIT-AGEING study. *Submitted*.

14 **Amaro-Gahete FJ**, De-la-O A, Jurado-Fasoli L, Espuch-Oliver A, de Haro T, Gutiérrez A *et al*. Exercise training increases the S-Klotho plasma levels in sedentary middle-aged adults: a randomised controlled trial. ***J Sport Sci*** 2019. In press.

15 **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. In press.

16 **Amaro-Gahete FJ**, De-la-O A, Jurado-Fasoli L, Dote-Montero M, Gutiérrez A, Ruiz J *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 randomised controlled trial. ***Front Physiol*** 2019. In press.

17 **Amaro-Gahete FJ,** De-la-O A, Jurado-Fasoli L, Sanchez-Delgado G, Ruiz J, Castillo M. Basal metabolic rate and fat oxidation in basal conditions and during exercise in sedentary middle-aged adults, following different exercise training interventions: a randomised controlled trial THE FIT AGEING Study. *Submitted*.

18 **Amaro‐Gahete FJ**, De-la-O A, Jurado-Fasoli L, Martinez-Tellez B, Ruiz J, Castillo J. Exercise training as a treatment for cardiometabolic risk in sedentary middle-aged adults: are physical activity guidelines the best way to improve cardiometabolic health? The FIT-AGEING randomized controlled trial. *Submitted*.

ABSTRACT

Since the discovery of the Klotho gene as a suppressor of several ageing phenotypes, numerous studies have focussed on elucidating the molecular pathways that mediate the effects of its expression on cellular ageing-related processes. However, the role of the shed form of the Klotho protein on physical fitness, energy metabolism and cardiometabolic health has not been deeply studied. Moreover, there is a biological base supporting the hypothesis that physical exercise could induce an increment of S-Klotho, resulting in one of the still unrecognized physiological mechanism that can explain the exercise benefits on the ageing process.

The main aims of this International Doctoral Thesis are to study the association of S-Klotho with physical fitness, energy metabolism and cardiometabolic health, and to study the effect of different exercise training programs on S-Klotho, as well as on physical fitness, energy metabolism, and cardiometabolic health in sedentary middle-aged adults.

The results show that lean mass is strongly associated with S-Klotho and explains the association of physical fitness with S-Klotho. BFox and MFO are positively associated with S-Klotho, whereas S-Klotho is negatively associated with cardiometabolic risk. Moreover, exercise training (specially a HIIT+EMS program) induces an increase of S-Klotho, improves body composition, physical fitness and energy metabolism, and reduces cardiometabolic risk.

In summary, the results show that S-Klotho plays a key role on physical fitness, energy metabolism and cardiometabolic health in sedentary middle-aged adults, and that exercise training modulates S-Klotho, as well as physical fitness, energy metabolism and cardiometabolic health. These findings may partially explain some of the uknown exercise-induced effects on cardiometabolic health as well as on the human ageing process.

RESUMEN

Tras el descubrimiento del gen Klotho como un supresor de distintos fenotipos asociados al envejecimiento, numerosos estudios se han centrado en investigar los mecanismos moleculares que median los efectos de su expresión sobre procesos relativos al envejecimiento celular. Sin embargo, no se ha estudiado en profundidad el rol que ejerce la proteína Klotho en su forma soluble sobre el metabolismo energético, la función cardiometabólica y la salud general en humanos. Además, hay una base biológica que permite hipotetizar que el ejercicio físico podría inducir un incremento de S-Klotho, pudiendo ser este uno de los mecanismos aun no conocidos que podrían explicar los ya extensamente reportados beneficios del ejercicio físico sobre la salud en humanos.

Los principales objetivos de la presente Tesis Doctoral Internacional son estudiar la asociación de S-Klotho con la condición física, el metabolismo energético y la función cardiometabólica, e investigar el efecto de distintos programas de ejercicio físico sobre S-Klotho, condición física, metabolismo energético y función cardiometabólica.

Los resultados muestran que la masa magra se asocia a S-Klotho y que dicha masa magra explica la asociación observada entre la condición física y S-Klotho. La oxidación de grasas en condiciones basales y la máxima oxidación de grasas durante el ejercicio se asocian positivamente con S-Klotho, mientras que S-Klotho se asocia negativamente con el riesgo cardiometabólico. Además, el ejercicio físico (especialmente un programa de HIIT+EMS) incrementa los niveles de S-Klotho, además de provocar una mejora de la composición corporal, la condición física y el metabolismo energético, y reducir el riesgo cardiometabólico.

En resumen, los resultados ponen de manifiesto que el ejercicio físico mejora S-Klotho, la condición física, el metabolismo energético y la salud cardiovascular en adultos sedentarios de mediana edad. Estos resultados podrían explicar parcialmente algunos de los efectos no conocidos del ejercicio sobre la salud cardiovascular y sobre el proceso de envejecimiento humano.

INTRODUCTION

Chapter 1:
Health-related benefits of exercise during the ageing process

AGEING POPULATIONS: THE CHALLENGES OF THE FUTURE

The considerable increment of ~30 years in life expectancy in western Europe, the United States of America, Canada, Australia, or New Zealand— and even greater gains in Japan, Spain or Italy—stands out as one of the most important achievements of the last century [1]. However, recent studies have suggested that chronological age is but a crude indicator of ageing. Therefore, specific ageing biomarkers have been proposed as providing a more accurate picture of the human health during the ageing process [2].

Ageing is a complex and multifactorial process influenced by both genetic and environmental factors, and characterised by a progressive decline of physiological functions, which leads to an impaired physical integrity and an increase of mortality risk [3,4]. There are a number of age-related diseases including metabolic and cardiovascular diseases, bone disorders, neurodegenerative diseases, or cancer, among others [5,6]. This increment of the age-related diseases has caused a public health and economic burden [5,6].

Ageing should not be considered as a disease, since it is a natural, physiological, progressive, and unavoidable process that can be influenced [7]. In this context, a new concept has appeared: "successful ageing" [7]. This refers to slowing down the functional decline and preventing diseases related to ageing. The aim would not be to add years to life, but to add life to years [8]. That is not to prolong life, but to living a full and active life for as long as possible. Several proposals have been made to support the "successful ageing" concept, including pharmacological [9], nutritional [10], and physical activity [11] interventions.

PHYSICAL EXERCISE AND FITNESS AS A PROMISING TOOL TO PROMOTE HEALTHY AGEING

It is well known that regular physical exercise exerts an important role in the ageing process. Previous studies suggested that doing physical exercise with an adequate intensity and duration could contribute to maintain or even improve the physical fitness level, obtaining greater anti-ageing effects [12–14].

Physical fitness is the ability to do physical activity and/or physical exercise using most of the body structures and functions involved in body movements such as the musculoskeletal, cardiorespiratory, hemato-circulatory, endocrine-metabolic system, among others [12–14].

It is well known that humans suffer a progressive physiological and functional decline (i.e. 10% per decade). A previous study showed that the maximum functional capacity occurs between the ages of 20 and 30 approximately, and the clinical manifestations of functional failure occur when 80% of the functional capacity has been depleted [15]. We can estimate that, in well conditions, excellent health could be maintained until the age of 100 years. Two decades later, the exhaustion of functional capacity would be generalized. Reducing the slowdown of the functional capacity to 8-9% would be a real and effective anti-ageing therapy. Consequently, measuring the level of physical fitness as a method to determine the functional capacity, health status, expectancy, and quality of life is of great importance [16].

VO_2max is the main indicator of cardiorespiratory fitness. There is highly variability among studies, but the average rate of VO_2max decline in old people is 4-5 ml/kg/min per decade. Ageing skeletal muscles have a poor capacity to use O_2 as a consequence of several factors, including a decrement of muscle mass, an increment of peripheral resistance, a reduced muscle capillary density, a low endothelial function, and an impaired muscle oxidative capacity [14,17].

The decline of muscle mass usually begins after 30 years of age. The main responsible is the loss of muscle cross-sectional area, which produces a progressive decrease of muscle strength with advancing age. The term ''sarcopenia'' was originally created to refer to age-related loss of muscle mass with an associated loss of muscular strength. Several age-related factors are associated with sarcopenia, including a progressive muscle denervation, diminished satellite cells, poor muscle protein synthesis, decrements of anabolic hormone levels, malnutrition, higher concentrations of pro-inflammatory cytokines, increased oxidative stress, mitochondrial impairments, and low levels of physical activity.

Regular physical exercise - concretely endurance training involving aerobic energy pathway and large muscular groups - attenuates ageing-related declines in cardiorespiratory fitness. It has a restoring effect on several cardiometabolic risk factors, increasing endothelial nitric oxide production and, consequently, improving vascular tone regulation. Similarly, this physical activity modality improved laminar flow activate endothelial nitric oxide synthase attenuating the production of reactive oxygen and nitrogen species.

Resistance exercise training programs are usually used as an effective strategy for improving muscle mass and/or muscular strength in the elderly producing a better (i) muscle quality, (ii) balance and mobility, (iii) motor performance and control, (iv) flexibility and joint range of motion, and (v) O2 arterio-venous difference.

Therefore, to maintain an adequate level of physical fitness performing physical exercise could be considered as a real anti-ageing strategy.

ENERGY METABOLISM AND CARDIOMETABOLIC RISK DURING THE AGEING PROCESS: ROLE OF PHYSICAL EXERCISE

The ageing process is accompanied by a progressive decline of total energy expenditure including REE, dietary-induced thermogenesis and activity energy expenditure [18]. These changes are closely related to weight gain and an increment of FM, which are closely related to the development of chronic cardiometabolic diseases, musculoskeletal and neuro-degenerative disorders, and/or Alzheimer's disease. It has been proposed that impairments of fuel oxidation in different conditions (i.e. basal, postprandial and exercise-induced fuel oxidation) could be important physiological mechanism contributing to age-related chronic diseases.

Physical exercise induces changes in the epigenome, transcriptome, and proteome to support increased storage of fuel and increased ability to use different energetic substrates depending on the environmental conditions. Physical exercise can promote better rates of fat oxidation at rest and during acute exercise [19]. In this sense, dual effects have been attributed to physical exercise including an enhancement of insulin sensitivity and a likely downstream benefit of reducing type II diabetes mellitus and cardiometabolic disease risk.

In conclusion, physical exercise is not able to reverse the ageing process, but it can ttenuate several of its deleterious systemic and cellular effects

REFERENCES

1 Christensen K, Doblhammer G, Rau R, Vaupel JW. Ageing populations: the challenges ahead. *Lancet (London, England)* 2009; **374**: 1196–208.

2 Foo H, Mather KA, Thalamuthu A, Sachdev PS. The many ages of man: diverse approaches to assessing ageing-related biological and psychological measures and their relationship to chronological age. *Curr Opin Psychiatry* 2019; **32**: 130–137.

3 López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. *Cell* 2013; **153**.

4 Sen P, Shah PP, Nativio R, Berger SL. Epigenetic mechanisms regulating longevity and aging. *Cell* 2016; **166**: 822–39.

5 Jin K, Simpkins JW, Ji X, Leis M, Stambler I. The Critical Need to Promote Research of Aging and Aging-related Diseases to Improve Health and Longevity of the Elderly Population. *Aging Dis* 2015; **6**: 1.

6 Fulop T, Larbi A, Witkowski JM, McElhaney J, Loeb M, Mitnitski A *et al.* Aging, frailty and age-related diseases. *Biogerontology* 2010; **11**: 547–563.

7 Bloom M, Klein W. *Successful aging: strategies for healthy living*. Springer Science & Business Media, 2013.

8 Havighurst R. *Process of aging: social and psychological perspectives*. 2008.

9 Gruber J, Halliwell B. Approaches for extending human healthspan: from antioxidants to healthspan pharmacology. *Essays Biochem* 2017; **61**: 389–399.

10 Crogan NL. Nutritional Problems Affecting Older Adults. *Nurs Clin North Am* 2017; **52**: 433–445.

11 Rebelo-Marques A, Lages ADS, Andrade R, Ribeiro CF, Mota-Pinto A, Carrilho F *et al.* Aging hallmarks: The benefits of physical exercise. *Front Endocrinol (Lausanne)* 2018; **9**: 1–15.

12 Pedersen B, Saltin B. Exercise as medicine - Evidence for prescribing exercise as therapy in 26 different chronic diseases. *Scand J Med Sci Sport* 2015; **25**: 1–72.

13 Castillo-Garzón MJ, Ruiz JR, Ortega FB, Gutiérrez A. Anti-aging therapy through fitness enhancement. *Clin Interv Aging* 2006; **1**: 213–20.

14 Amaro Gahete FJ, De La O A, Jurado Fasoli L, Castillo MJ, Gutierrez A. Fitness Assessment as an Anti-Aging Marker: A Narrative Review. *J Gerontol Geriatr Res* 2017; **06**.

15 Moody H, Sasser J. *Aging : concepts and controversies. Pine Forge Press.* 1994.

16 Myers J, McAuley P, Lavie C, Despres J, Arena R, Kokkinos P. Physical activity and cardiorespiratory fitness as major

markers of cardiovascular risk: their independent and interwoven importance to health status. *Prog Cardiovasc Dis* 2015; **57**: 306–314.

17 Garatachea N, Pareja-Galeano H, Sanchis-Gomar F, Santos-Lozano A, Fiuza-Luces C, Morán M *et al.* Exercise Attenuates the Major Hallmarks of Aging. *Rejuvenation Res* 2015; **18**: 57–89.

18 Siervo M, Lara J, Celis-Morales C, Vacca M, Oggioni C, Battezzati A *et al.* Age-related changes in basal substrate oxidation and visceral adiposity and their association with metabolic syndrome. *Eur J Nutr* 2016; **55**: 1755–67.

19 van Loon LJ, Jeukendrup AE, Saris WH, Wagenmakers AJ. Effect of training status on fuel selection during submaximal exercise with glucose ingestion. *J Appl Physiol* 1999; **87**: 1413–1420.

Chapter 2:
Role of exercise on S-Klotho protein regulation: a systematic review
(Study 1)

ABSTRACT

Humans have long sought means to extend longevity and counteract the effects of aging on physical and mental functioning. Exercise is a highly effective way for treating and preventing the main causes of morbidity and mortality, most of which are associated with aging. Interestingly, the Klotho gene is involved in the aging process. Indeed, overexpression of the Klotho gene is associated to longevity, and experimental animals lacking this gene seem to develop multiple disorders resembling human aging, and present a shortened life span. Three Klotho related gene have been identified: α-Klotho, β-Klotho, and γ-Klotho. Exercise seems to play a key role on α-Klotho gene expression in animal models as well as in humans. We systematically reviewed the available evidence on the associations between exercise and α-Klotho gene expression.

BACKGROUND

The human race has long sought means to extend longevity and counteract the effects of ageing on physical and mental functioning. Considerable strides have been made in our biological understanding of the factors contributing to the ageing process. This knowledge is crucial for the development of therapeutic and clinical strategies to prevent, delay, or reverse age-related decline [1].

Expression of the Klotho gene [named after one of the three Fates in Greek mythology, the goddess who spins the thread of life] is involved in the ageing process. It was originally identified in a mutant mouse strain lacking the expression of Klotho. This defect caused no visible phenotypes until 3-4 weeks of age, but the mice subsequently developed multiple disorders resembling human ageing and presented a shortened lifespan [2]. The ageing phenotypes included arteriosclerosis, decreased BMD, sarcopenia, skin atrophy, and impaired cognition [3–6]. In contrast, Klotho overexpression produced a significantly longer lifespan in transgenic mice compared with their wild-type peers [3,4,6].

Three Klotho-related genes have been identified: (i) α-Klotho is expressed in multiple tissues and cell types, predominantly in distal convoluted tubules in the kidney, parathyroid and choroid plexus in the brain, and it is involved in the control of mineral homeostasis [2]. (ii) β-Klotho is expressed in the liver, endocrine pancreas, adipose tissue and brain, and it regulates bile acids, lipid and energy metabolism together with FGF15/19 and FGF21 [7–9]. (iii) γ-Klotho is a half-size Klotho-related protein expressed in brown adipose tissue [10,11].

The α-Klotho gene can be expressed as three different forms [10]: (i) intra-cellular form, which binds NaK-ATPase, (ii) cell-membrane form, which forms a complex with FGF23 and FGFR1, and (iii) S-Klotho, identified in blood, plasma, urine, and cerebrospinal fluid [12]. The secreted form of α-Klotho is composed of two internal repeats, KL1 and KL2, which share amino-acid sequence homology with β-glucosidase but lack glucosidase activity. KL1 could also be transcribed through an alternative splicing, named S-Klotho [2]. Unfortunately, the lack of a sensitive and reliable assay for measuring blood concentrations of the S-Klotho has hindered research into the relationship of S-Klotho protein levels with clinical phenotypes in human ageing. However, several trials have determined a sensitive and specific method for the measurement of S-Klotho protein levels in healthy humans [13–15].

Several studies have demonstrated the effectiveness of exercise to prevent, delay, or reverse the effects of ageing on tissue health and functioning. These systemic anti-ageing benefits suggest that humoral factors may play a role, especially in sarcopenia treatment [16]. Moreover, it is well known that exercise has a positive impact and attenuates premature ageing. Among its benefits we can find greater walking capacity [17], higher BMD concentration [18], and cognitive function improvements [19]. Furthermore, it is positively associated with regenerative tissue response [20], lower incidence of atherosclerosis [21], type 1 diabetes mellitus [22], and kidney cancer risk [23], attenuating premature ageing.

Likewise, higher concentrations of S-Klotho are associated with a superior lower extremity strength and functioning [24], a reduced likelihood of developing Alzheimer´s disease [25], and an increment of re-myelination of the brain in sclerosis patients [26]. Furthermore, high concentrations are also associated with resistance to oxidative stress [27], an increase of stem cell numbers and regenerative response [28], a lower apoptosis incidence in pancreatic β cells [29], a reduced incidence of renal fibrosis and cancer metastasis [30], and a lower risk of cardiovascular disease and mortality [31].

Exercise is considered a highly effective mean of treating and preventing the main causes of morbidity and mortality, most of which are associated with ageing [32]. Therefore, the objective of this systematic review was to study the available evidence on the associations between exercise and S-Klotho protein regulation.

MATERIAL & METHODS

Search strategy

We conducted a systematic review of the literature using a pre-specified protocol according to the guidelines of the Cochrane Collaboration and PRISMA recommendations [33]. We included cross-sectional studies and randomized and non-randomized controlled trials written in English. We excluded the uncontrolled studies. No exclusion criteria were applied to participants. Therefore, our study included healthy, untrained, trained, sedentary, recreational and non-athletic, athletes, patients with acute of chronic diseases aged between 18 and 90 years old, and animal models providing information about (i) acute and chronic effect of different exercise modalities on S-Klotho protein levels and (ii) the relationship between physical fitness and S-Klotho protein levels.

Data sources and study search

We searched in PubMed, Web of Science, SPORTDiscuss, EMBASE, CINAHL, Google Scholar, and the Cochrane library using all available records up to June 2017. The search terms covered the areas of Klotho genes, Klotho proteins, exercise, and physical fitness using combinations of the following key words: Klotho, S-Klotho, α-Klotho, β-Klotho, γ-Klotho, exercise, physical activity, fitness, physical fitness, skeletal muscle, strength, activities day-living, aerobic, anaerobic, endurance, training, and health. Two authors (FAG and AOP) independently conducted the literature search, quality assessment, and data extraction. We excluded all papers that did not meet the inclusion criteria (see below). Inter-reviewer disagreements were resolved by consensus opinion or arbitration by a third reviewer (AG). Then, we collected full papers, including reviews, and we also examined the reference lists of the selected manuscripts for any other potentially eligible papers. Figure 1 includes the full search strategy and protocol for the systematic review.

Outcomes

Inclusion criteria: Studies analyzing the association of S-Klotho protein levels with exercise and/or physical fitness; Studies conducted in humans and/or mice; Cross-sectional, longitudinal, or intervention studies; Written in English or Spanish. We also registered the following related outcomes: (i) changes in skeletal muscle, changes in strength, changes in arterial stiffness, and changes in running endurance. We collected data on age, sex, exercise modality and duration, study location as well as the inclusion and exclusion criteria of each study.

RESULTS AND DISCUSSION

The initial search strategy found 1,278 results to be considered for inclusion in our systematic review. Of these results, 168 duplicates were excluded, 91 were not full-text, 67 were not available in the English language, and 21 were meta-analyses or review articles. A total of 931 articles were screened based on their title and abstract (859 and 49 were excluded, respectively). We then evaluated in detail the full-text of 23 articles. As a result, 14 studies were included in the systematic review. The reasons for exclusion in this final phase included the studies that did not analyze the association between S-Klotho protein level measurements and exercise or physical activity application or physical fitness measurements. Table 1 displays the study details. We found seven studies that included the effect of acute and chronic effects of different training modalities on S-Klotho protein levels and five studies that examined the possible relationship between physical fitness and S-Klotho protein levels.

Klotho functions

The α-Klotho gene can be expressed as three functionally different family members (Figure 2). Intracellular Klotho is present in the cytoplasm of mouse kidney and human parathyroid gland cells [10]. Interestingly, it is involved in intracellular calcium modulation, wherein an aberrant cellular and subcellular control of calcium can affect the ageing process in different tissues. Intracellular Klotho binds NA+/K+-ATPase and stimulates its surface abundance and activity, thereby providing a driving force for transmembrane Ca^{2+} transport. This has been clearly demonstrated in the choroid plexus and kidney [10].

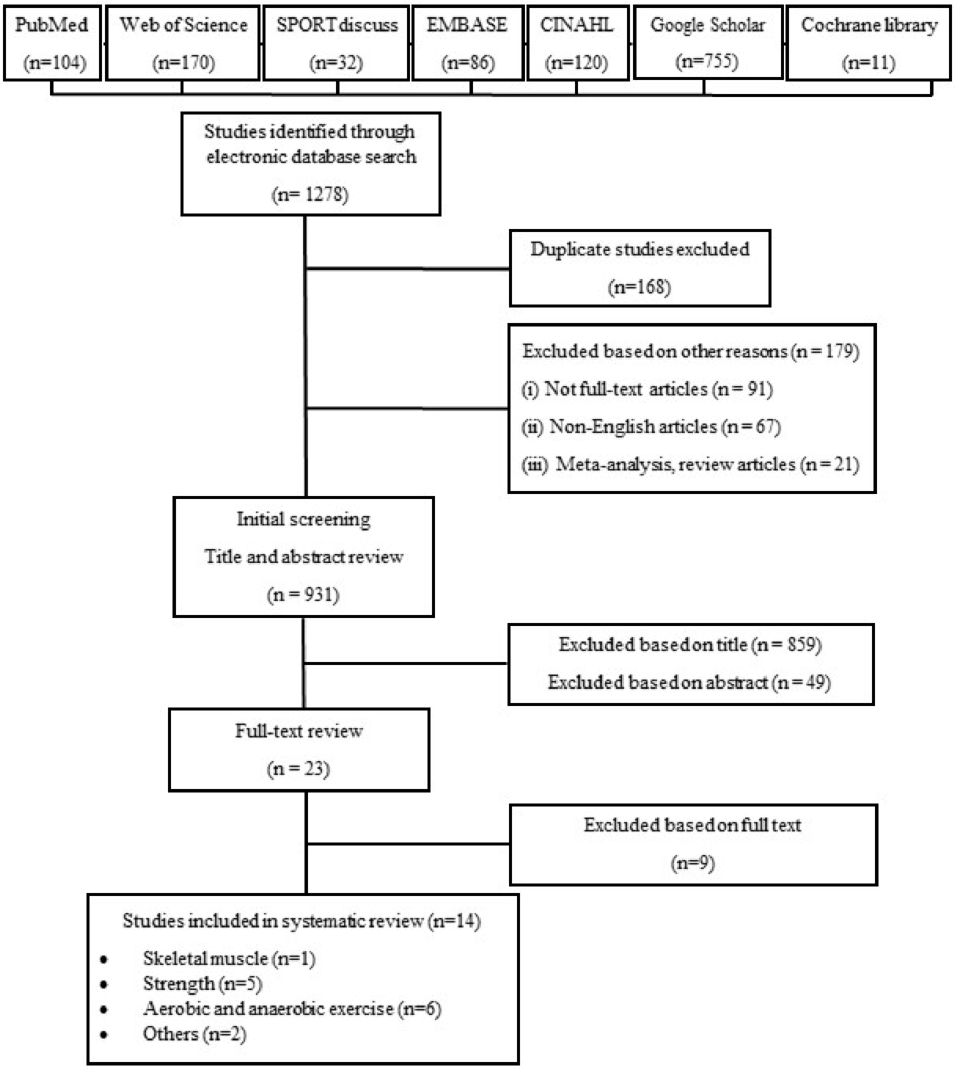

Figure 1. Literature search flow diagram

Table 1. Characteristics of the selected studies and intervention details

Study	N	Study population	Age (y)	Study duration	Key-findings
Avin et al. (2014)	Not reported	Young and aged C57Bl6/J mice	3-4 months vs 22-24 months	Cross-sectional study	A significant increase in S-Klotho protein levels in both young and aged mice, although the response was blunted in aged animals when compared to their young counterparts
Phelps et al. (2013)	Not reported	Mice	Not reported	Cross-sectional study	S-Klotho protein deficiency influences muscle strength and running endurance in mice
Avin et al. (2014)	19	Young vs older sedentary individuals	36±7 vs. 68±3	Cross-sectional study	No significant changes in S-Klotho protein levels in response to an acute exercise bout pre-to-post in young and older individuals
Avin et al. (2014)	19	Young vs older sedentary individuals	36±7 vs 68±3	16 weeks	A significant increase in S-Klotho protein levels in response to an acute exercise bout in young and older individuals (attenuated when compared with young) after completing a 16-week training program
Baldan et al. (2015)	201	Patients with β-thalassemia major vs healthy adults	38.6±6.5 vs. 40.9±7.8	Cross-sectional study	S-Klotho protein levels lower than 520 pg/ml increased the probability of fractures by nearly 4-folds and a correlation between S-Klotho protein levels and hand-grip strength (up to 580 ± 149 pg/ml) in patients with β-thalassemia was found
Crasto et al. (2012)	802	Older healthy adults	>65	Cross-sectional study	Low S-Klotho protein levels were independently associated with activities of daily living disability among older community-dwelling men and women
Matsubara et al. (2014)	69	Healthy and postmenopausal women	60±1	12 weeks	(i) S-Klotho protein levels positively correlated with carotid artery compliance and oxygen uptake at ventilatory threshold and negatively correlated with the β-stiffness index. (ii) Aerobic exercise training increased S-Klotho protein levels and carotid artery compliance and decreased the β-stiffness index
Mostafidi et al. (2016)	58	Healthy football players vs healthy young adults	18-22 vs. 18-27	Cross-sectional study	Regular aerobic exercise could increase S-Klotho protein levels, and this could be an explanation for exercise-related anti-ageing effects
Saghiv et al. (2015a)	200	Healthy young active, inactive, and trained males and healthy elderly active, inactive, and trained males	23.9±1.0 vs. 58.1±1.1	Cross-sectional study	S-Klotho protein levels are associated with younger age and aerobic exercise training performance
Saghiv et al. (2015b)	30	Elite anaerobically trained sprinters and elite aerobically well-trained athletes	24.4±1.0 vs. 24.7±1.0	Cross-sectional study	S-Klotho protein levels and long-lasting aerobic exercise training are factors that may promote upgrading capacities of young adults. However, there is no association between anaerobic vigorous exercise training and decreased risk factors for major chronic diseases

Santos-Dias et al. (2016)	21	Healthy young trained adults	34.8±1.8.9	Cross-sectional study	A single maximal aerobic exercise session of 20 min of duration induced an increase of S-Klotho protein levels, particularly in women
Semba et al. (2012)	775	Older healthy adults	>65	Cross-sectional study	S-Klotho protein levels were associated with grip in adults with S-Klotho protein levels <681 pg/mL
Semba et al. (2016)	2,734	Older healthy adults	74.5±2.9	Cross-sectional study	S-Klotho protein levels were an independent predictor of changes in knee strength over time in older adults.
Shardell et al. (2015)	860	Older healthy adults	>55	Cross-sectional study	S-Klotho protein levels and 25(OH)D were both positively related to lower-extremity physical performance

Moreover, intracellular Klotho was also recently found to block the RIG-I, which is responsible for the increased expression of pro-inflammatory cytokines such as IL-6 and IL-8 [34]. Therefore, the fact that the expression of these cytokines is associated with senescence suggests that intracellular Klotho could function as an intracellular anti-inflammatory and anti-ageing factor [10].

In addition, cell-membrane of α-Klotho is a constitutive part of the FGFR and its ligands are the members of the endocrine FGF family. This family is present in humans by FGF19 [FGF15 in mice], FGF21, and predominantly FGF23, which is a bone-derived hormone that acts on the kidney to promote phosphate excretion into the urine and regulates Ca2+ homeostasis [10]. Specifically, the amount of urinary phosphate excretion is primarily determined by the amount of phosphate reabsorbed at the renal proximal tubules, which depends on the activity of NaPi-2ab expressed on the apical brush border membrane of these tubules [35–37]. Of note, FGF23 contributes to the onset of phosphaturia by suppressing NaPi-2a activity [36,37]. Mice lacking FGF23 (FGF23-/-) develop phosphate-retention phenotypes and exhibit unexpected phenotypes.

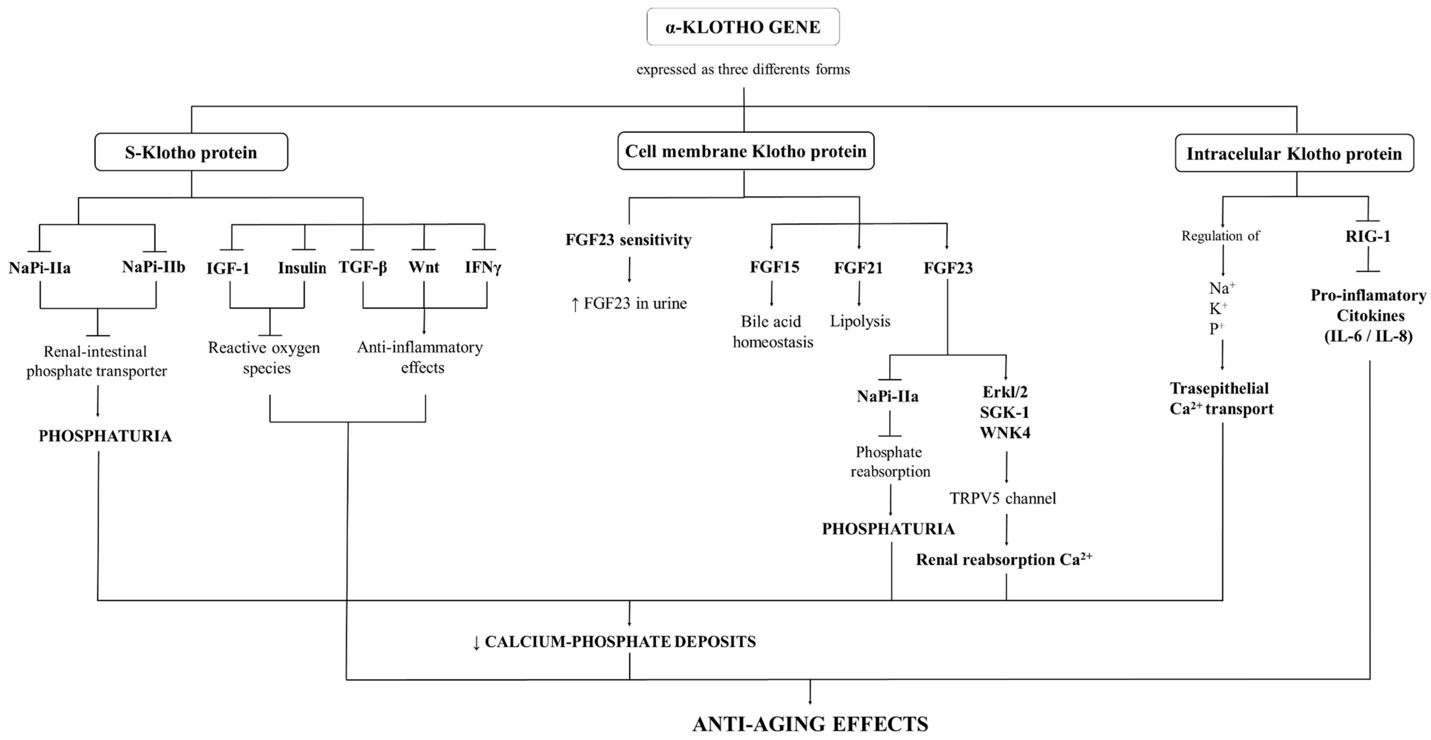

Figure 2. General and specific functions of expressions of the three forms of the α-Klotho gene and the possible relationships with anti-ageing factors

These include growth retardation, hypogonadism, premature thyme involution, sarcopenia, osteopenia, skin atrophy, and pulmonary emphysema, which are reminiscent of the premature-ageing syndrome in Klotho-deficient mice [38]. In support of this notion, Klotho-deficient mice have extremely high serum FGF23 levels, indicating that the loss of Klotho induces resistance to FGF23 [9]. In addition, FGF23 acting on the Klotho-FGFRs complexes at the basolateral side stimulates renal Ca2+ reabsorption via the TRPV5 channel, which is expressed in the apical membrane of the distal convoluted tubule. The Klotho-FGFR complex activates signalling cascades involving Erk1/2, SGK-1, and WNK4 for TRPV5-mediated Ca2+ reabsorption [39].

In particular, β-Klotho also contributes to the regulation of energy metabolism as an obligate co-receptor for FGF15 and FGF21 in rats [40,41]. The complex formed by β-Klotho and FGF15 is indispensable for maintaining bile acid homeostasis [42,43]. In contrast, FGF21 is secreted from the liver upon fasting and acts on adipose tissue to promote lipolysis [44]. Moreover, γ-Klotho, which is fundamentally expressed in brown adipose tissue, forms complexes with FGFR to increase FGF19 activity; however, its biological function remains elusive [10,11].

S-Klotho functions as a humoral factor that targets multiple tissues and organs [45]. S-Klotho exerts anti-ageing and organ protection effects [10] through the modulation of the action of growth factors and cytokines such as insulin, IGF-1, TGF-β, Wnt signaling, and IFNγ, which are associated with cell senescence and the ageing process in mice [10,30]. Indeed, several studies suggest that the over-expression of the α-Klotho gene down regulates the signalling of insulin and IGF-1, attenuating the generation of reactive oxygen species and thereby extending their lifespan [27,30]. In addition, Klotho-deficient mice that lack the anti-inflammatory effects of the S-Klotho protein display increased TNF-α, Wnt signalling, and IFNγ levels, which contributes to accelerated ageing and premature mortality in these models [29]. In summary, multiple studies have demonstrated that the S-Klotho protein might function as an anti-ageing and organ protection factor.

Finally, the S-Klotho protein maintains ion homeostasis by regulating ion channels and/or phosphate transporters. Indeed, high S-Klotho protein levels inhibit renal and intestinal phosphate transportation (NaPi-2a and NaPi-2b) avoiding phosphate reabsorption and producing phosphaturia independent of FGF23 [46]. In conclusion, the S-Klotho protein has a regulatory function in calcium-phosphate metabolism playing an important role in the prevention of chronic kidney disease [47].

Role of exercise on S-Klotho protein

In murine models, the S-Klotho protein levels are associated with endurance capacity and skeletal muscle strength in Klotho-overexpressing mice, wild-type mice, and Klotho-deficient C57BL/6 mice. Muscle strength of Klotho-hypomorphic mice was around 50% less than that of Klotho-overexpressing mice or wild-type mice [48]. Interestingly, Klotho-deficient mice ran on the running wheel at the same speed as the other two groups, but they spent about 65% less time running than Klotho-overexpressing and wild-type mice [48].

Another study assessed the effect of acute exercise bouts on S-Klotho protein levels in both young [3–4 months] and aged (22–24 months) C57Bl6/J mice and observed a clear effect on S-Klotho protein levels [1]. Particularly, mice performed an acute exercise consisting in 45-min of treadmill running at 70% of VO_2max and the results showed a significant increase in S-Klotho protein levels in both young and aged mice. However, the response was higher in young animals when compared to their aged counterparts [1].

In humans, interventional studies have been implemented in order to analyze the effects of a single acute exercise and an exercise training protocol on the S-Klotho protein levels of sedentary young adults and sedentary older adults. Particularly, the acute exercise bout for the young group consisted in one hour of treadmill walking at 55% of VO_2max. For the older group, it consisted in one hour on a cycloergometer at 45% of VO_2max. The exercise training protocol consisted in four to six exercise sessions weekly, which included cycling on a stationary bicycle, rowing, or walking/jogging at 55% of VO_2max (the young group during 16 weeks) and 45% of VO_2max (the older group during 14 weeks). Interestingly, before the intervention program, no significant changes in S-Klotho protein levels in response to an acute exercise bout pre-to-post was observed in young individuals. However, the completion of the 16-week training program significantly increased the S-Klotho protein levels, which also rose in response to the acute exercise bout in both young and old individuals. The response of S-Klotho protein levels to an acute aerobic exercise was higher after the training period, although the effect was lower on the older compared to the younger participants [1].

To study not only the age-related S-Klotho protein level changes, but also the effect of training status, an intervention study quantified the effect of an acute exercise bout on S-Klotho protein levels in both trained and untrained young (24.5±1.0 and 23.9±1.0 years old, respectively) and aged (58.6±1.1 and 58.1±1.1 years old, respectively) adults [49]. The acute exercise consisted in a graded maximal treadmill test. This study demonstrated that S-Klotho protein levels are significantly higher in healthy well-trained young and elderly subjects compared to their untrained counter-partners. Furthermore, well-trained young adults presented significantly higher values [49]. Consistently, a recent intervention study designed to determine the effect of aerobic exercise on S-Klotho protein levels in an experimental group of trained athletes (30 healthy football male players aged 18-22) and in a control group (28 healthy young males aged 18-27) showed that aerobic exercise training induced a net increase in plasma S-Klotho protein levels [50]. Unfortunately, the persistence of this increase over time was not investigated, given that concentrations were only measured at one-time point (day after exercise training) in the experimental group [50]. In this regard, the study revealed that only 20-minute bouts of maximal intensity running increased the S-Klotho protein levels in 21 healthy young adults who performed resistance and aerobic training for at least 1 year, 4 to 5 times per week [51].

In order to examine whether the S-Klotho protein levels depend on the type of exercise on well-trained young adults, an interventional study was implemented. The participants were 30 healthy young sportsmen, 15 well-trained sprinters at the national level (24.2±1.0 years old and 55.4±2.7 ml/kg/min VO_2max) and 15 aerobically well-trained elite runners (24.7±1.0 years old and 60.3±2.6 ml/kg/min VO_2max) [52]. In this study, an acute exercise consisting in 60 min of treadmill running at 75% of VO_2max showed that the S-Klotho protein levels were markedly higher in aerobic trained sportsmen compared to those measured in the anaerobic sprinters [52]. It also showed that sprinters and sedentary young adult males had similar S-Klotho protein levels [52], thus demonstrating that the S-Klotho protein response could be dependent on aerobic fitness level. However, few studies have been published regarding the influence of a training program on S-Klotho protein levels. In this context, a study of 19 healthy postmenopausal women (aged 50–76) explored the effect of regular exercise on S-Klotho protein levels [53]. The women who were assigned to 12 weeks of moderate aerobic exercise training showed an increase in S-Klotho, improvement in carotid artery compliance, and a decrease in β-stiffness index. However, no changes were observed in the control group over the same time period [53].

S-Klotho protein and physical fitness

Limited data are available on the relationship between physical fitness, physical health, and the S-Klotho protein (Figure 3).

Physical fitness is considered a measure of the ability to perform physical activity/exercise, and it involves most body structures and functions (locomotor function, cardiorespiratory, blood-circulatory, endocrine-metabolic, psychological, neurological system/apparatus, etc.) [32].

Physical fitness is an excellent predictor of life expectancy [54], both for those who are healthy and for those with some form of cardiovascular disease [55,56]. Therefore, it is essential to monitor the level of fitness during the ageing process through the regular assessment of its components.

Interestingly, the InCHIANTI study demonstrated that many components of physical fitness are related to S-Klotho [24,57,58].

Furthermore, another recent study demonstrated a positive correlation between S-Klotho and aerobic capacity in postmenopausal women after a 12-week exercise program [53].

An interventional study observed poor grip strength in older community-dwelling adults with low S-Klotho [57], consistent with the presence of sarcopenia described in the Klotho mice model of ageing [4,28,47]. A negative correlation was also reported between hand-grip test and S-Klotho protein levels in β-thalassemia patients [59]. In addition, a greater decline in knee strength was observed over a 4-year follow-up period in older adults with lower versus higher S-Klotho [60].

Finally, a recent study showed a positive association between S-Klotho and lower-extremity physical performance [58] using the Short Physical Performance Battery derived from lower-extremity performance tests used in the Established Populations for the Epidemiologic Studies of the Elderly [61]. These findings are consistent with the interpretation of S-Klotho protein as a fitness and health marker in animal models [1,48] and in humans [1,49,53].

CONCLUSIONS

In conclusion, the S-Klotho protein may exert multiple anti-ageing functions, including regulatory functions in the calcium-phosphate metabolism, avoiding precipitation of calcium phosphate, reducing apoptotic mechanisms, inflammatory processes, and oxidative stress, and protecting cells. Despite this, future interventions are essential to explain the specific physiological mechanisms underlying these phenomena.

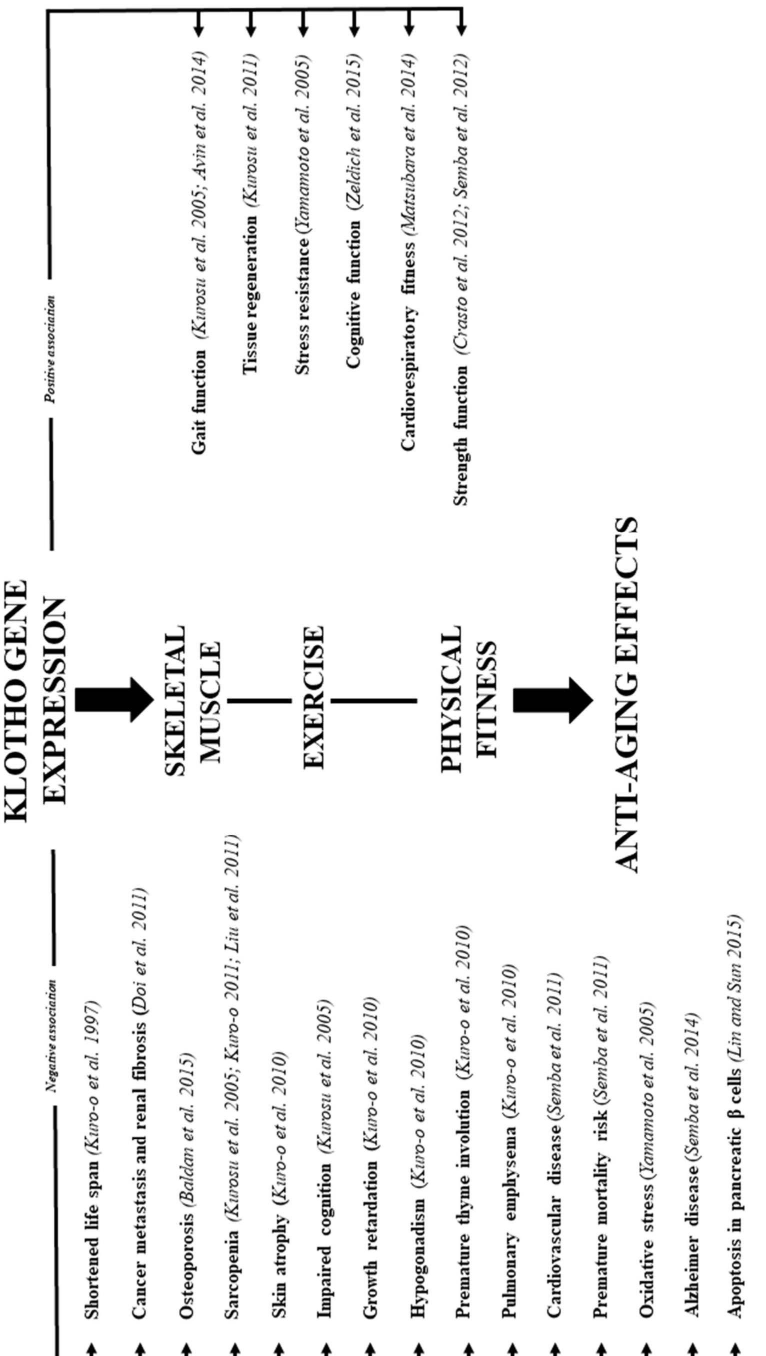

Figure 3. Klotho gene expression and concordant effects of physical exercise/fitness on ageing and wellbeing

REFERENCES

1 Avin KG, Coen PM, Huang W, Stolz DB, Sowa GA, Dubé JJ *et al.* Skeletal muscle as a regulator of the longevity protein, Klotho. *Front Physiol* 2014; **5**: 189.

2 Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T *et al.* Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* 1997; **390**: 45–51.

3 Kuro-o M. Klotho. *Pflugers Arch Eur J Physiol* 2010; **459**: 333–343.

4 Kurosu H, Yamamoto M, Clark JD, Pastor J V, Nandi A, Gurnani P *et al.* Suppression of aging in mice by the hormone Klotho. *Science* 2005; **309**: 1829–33.

5 Iida R, Kanko S, Suga T, Morito M, Yamane A. Autophagic-lysosomal pathway functions in the masseter and tongue muscles in the klotho mouse, a mouse model for aging. *Mol Cell Biochem* 2011; **348**: 89–98.

6 Saito Y, Yamagishi T, Nakamura T, Ohyama Y, Aizawa H, Suga T *et al.* Klotho protein protects against endothelial dysfunction. *Biochem Biophys Res Commun* 1998; **248**: 324–9.

7 Ben-Dov IZ, Galitzer H, Lavi-Moshayoff V, Goetz R, Kuro-o M, Mohammadi M *et al.* The parathyroid is a target organ for FGF23 in rats. *J Clin Invest* 2007; **117**: 4003–8.

8 Hu MCM, Shi M, Zhang J, Pastor J, Nakatani T, Lanske B *et al.* Klotho: a novel phosphaturic substance acting as an autocrine enzyme in the renal proximal tubule. *FASEB J* 2010; **24**: 3438–3450.

9 Urakawa I, Yamazaki Y, Shimada T, Iijima K, Hasegawa H, Okawa K *et al.* Klotho converts canonical FGF receptor into a specific receptor for FGF23. *Nature* 2006; **444**: 770–774.

10 Kim J-H, Hwang K-H, Park K-S, Kong I, Cha S-K. Biological Role of Anti-aging Protein Klotho. *J lifestyle Med* 2015; **5**: 1–6.

11 Tacer K, Bookout AAL, Ding XX, Kurosu H, John GBG, Wang L *et al.* Research resource: comprehensive expression atlas of the fibroblast growth factor system in adult mouse. *Mol Endocrinol* 2010; **24**: 2050–2064.

12 Olejnik A, Franczak A, Krzywonos-Zawadzka A, Kałużna-Oleksy M, Bil-Lula I. The Biological Role of Klotho Protein in the Development of Cardiovascular Diseases. *Biomed Res Int* 2018; **2018**: 1–17.

13 Devaraj S, Syed B, Chien A, Jialal I. Validation of an immunoassay for soluble Klotho protein: decreased levels in diabetes and increased levels in chronic kidney disease. *Am J Clin Pathol* 2012; **137**: 479–85.

14 Yamazaki Y, Imura A, Urakawa I, Shimada T, Murakami J, Aono Y *et al.* Establishment of sandwich ELISA for soluble alpha-Klotho measurement: age-dependent change of soluble alpha-Klotho levels in healthy subjects. *Biochem Biophys Res Commun* 2010; **398**: 513–518.

15 Pedersen L, Pedersen SM, Brasen CL, Rasmussen LM. Soluble serum Klotho

levels in healthy subjects. Comparison of two different immunoassays. *Clin Biochem* 2013; **46**: 1079–1083.

16 Montero-Fernández N, Serra-Rexach JA. Role of exercise on sarcopenia in the elderly. *Eur J Phys Rehabil Med* 2013; **49**: 131–43.

17 Simonsick EM, Fan E, Fleg JL. Estimating cardiorespiratory fitness in well-functioning older adults: treadmill validation of the long distance corridor walk. *J Am Geriatr Soc* 2006; **54**: 127–32.

18 Martyn-St James M, Carroll S. A meta-analysis of impact exercise on postmenopausal bone loss: the case for mixed loading exercise programmes. *Br J Sport Med* 2009; **43**: 898–908.

19 Foster PP, Rosenblatt KP, Kuljiš RO. Exercise-Induced Cognitive Plasticity, Implications for Mild Cognitive Impairment and Alzheimer's Disease. *Front Neurol* 2011; **2**.

20 Shefer G, Rauner G, Yablonka-Reuveni Z, Benayahu D. Reduced satellite cell numbers and myogenic capacity in aging can be alleviated by endurance exercise. *PLoS One* 2010; **5**: e13307.

21 Palmefors H, DuttaRoy S, Rundqvist B, Börjesson M. The effect of physical activity or exercise on key biomarkers in atherosclerosis--a systematic review. *Atherosclerosis* 2014; **235**: 150–61.

22 Slentz CA, Tanner CJ, Bateman LA, Durheim MT, Huffman KM, Houmard JA *et al.* Effects of exercise training intensity on pancreatic beta-cell function. *Diabetes Care* 2009; **32**: 1807–11.

23 Williams PT. Reduced risk of incident kidney cancer from walking and running. *Med Sci Sports Exerc* 2014; **46**: 312–7.

24 Crasto CL, Semba RD, Sun K, Cappola AR, Bandinelli S, Ferrucci L. Relationship of low-circulating 'anti-aging' klotho hormone with disability in activities of daily living among older community-dwelling adults. *Rejuvenation Res* 2012; **15**: 295–301.

25 Semba RD, Moghekar AR, Hu J, Sun K, Turner R, Ferrucci L *et al.* Klotho in the cerebrospinal fluid of adults with and without Alzheimer's disease. *Neurosci Lett* 2014; **558**: 37–40.

26 Zeldich E, Chen C-D, Avila R, Medicetty S, Abraham CR. The Anti-Aging Protein Klotho Enhances Remyelination Following Cuprizone-Induced Demyelination. *J Mol Neurosci* 2015; **57**: 185–196.

27 Yamamoto M, Clark JD, Pastor J V., Gurnani P, Nandi A, Kurosu H *et al.* Regulation of Oxidative Stress by the Anti-aging Hormone Klotho. *J Biol Chem* 2005; **280**: 38029–38034.

28 Liu H, Fergusson MM, Castilho RM, Liu J, Cao L, Chen J *et al.* Augmented Wnt signaling in a mammalian model of accelerated aging. *Science* 2007; **317**: 803–6.

29 Lin Y, Sun Z. Antiaging Gene Klotho Attenuates Pancreatic β-Cell Apoptosis in Type 1 Diabetes. *Diabetes* 2015; **64**: 4298–4311.

30 Doi S, Zou Y, Togao O, Pastor J V., John

GB, Wang L *et al.* Klotho Inhibits Transforming Growth Factor-β1 (TGF-β1) Signaling and Suppresses Renal Fibrosis and Cancer Metastasis in Mice. *J Biol Chem* 2011; **286**: 8655–8665.

31 Semba RD, Cappola AR, Sun K, Bandinelli S, Dalal M, Crasto C *et al.* Plasma Klotho and Cardiovascular Disease in Adults. *J Am Geriatr Soc* 2011; **59**: 1596–1601.

32 Castillo-Garzón MJ, Ruiz JR, Ortega FB, Gutiérrez A. Anti-aging therapy through fitness enhancement. *Clin Interv Aging* 2006; **1**: 213–20.

33 Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JPA *et al.* The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. *J Clin Epidemiol* 2009; **62**: e1-34.

34 Liu F, Wu S, Ren H, Gu J. Klotho suppresses RIG-I-mediated senescence-associated inflammation. *Nat Cell Biol* 2011; **13**: 254–262.

35 Shimada T, Urakawa I, Yamazaki Y, Hasegawa H, Hino R, Yoneya T *et al.* FGF-23 transgenic mice demonstrate hypophosphatemic rickets with reduced expression of sodium phosphate cotransporter type IIa. *Biochem Biophys Res Commun* 2004; **314**: 409–14.

36 Segawa H, Yamanaka S, Ohno Y, Onitsuka A, Shiozawa K, Aranami F *et al.* Correlation between hyperphosphatemia and type II Na-P i cotransporter activity in klotho mice. *Am J Physiol Physiol* 2007; **292**: F769–F779.

37 Segawa H, Kawakami E, Kaneko I, Kuwahata M, Ito M, Kusano K *et al.* Effect of hydrolysis-resistant FGF23-R179Q on dietary phosphate regulation of the renal type-II Na/Pi transporter. *Pflügers Arch - Eur J Physiol* 2003; **446**: 585–592.

38 Kuro-o M. Klotho. *Pflugers Arch* 2010; **459**: 333–43.

39 Andrukhova O, Smorodchenko A, Egerbacher M, Streicher C, Zeitz U, Goetz R *et al.* FGF23 promotes renal calcium reabsorption through the TRPV5 channel. *EMBO J* 2014; : n/a-n/a.

40 Ogawa Y, Kurosu H, Yamamoto M, Nandi A, Rosenblatt KP, Goetz R *et al.* betaKlotho is required for metabolic activity of fibroblast growth factor 21. *Proc Natl Acad Sci* 2007; **104**: 7432–7437.

41 Kurosu H, Ogawa Y, Miyoshi M, Yamamoto M, Nandi A, Rosenblatt KP *et al.* Regulation of Fibroblast Growth Factor-23 Signaling by Klotho. *J Biol Chem* 2006; **281**: 6120–6123.

42 Ito S, Fujimori T, Furuya A, Satoh J, Nabeshima Y, Nabeshima Y. Impaired negative feedback suppression of bile acid synthesis in mice lacking βKlotho. *J Clin Invest* 2005; **115**: 2202–2208.

43 Inagaki T, Choi M, Moschetta A, Peng L, Cummins CL, McDonald JG *et al.* Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. *Cell Metab* 2005; **2**: 217–225.

44 Inagaki T, Dutchak P, Zhao G, Ding X,

Gautron L, Parameswara V *et al.* Endocrine Regulation of the Fasting Response by PPARα-Mediated Induction of Fibroblast Growth Factor 21. *Cell Metab* 2007; **5**: 415–425.

45 Lim K, Groen A, Molostvov G, Lu T, Lilley KS, Snead D *et al.* α-Klotho Expression in Human Tissues. *J Clin Endocrinol Metab* 2015; **100**: E1308–E1318.

46 Hu MC, Shi M, Zhang J, Quiñones H, Griffith C, Kuro-o M *et al.* Klotho Deficiency Causes Vascular Calcification in Chronic Kidney Disease. *J Am Soc Nephrol* 2011; **22**: 124–136.

47 Kuro-o M. Klotho and the Aging Process. *Korean J Intern Med* 2011; **26**: 113.

48 Phelps M, Pettan-Brewer C, Ladiges W, Yablonka-Reuveni Z. Decline in muscle strength and running endurance in klotho deficient C57BL/6 mice. *Biogerontology* 2013; **14**: 729–39.

49 Saghiv M, Goldhammer E, Sagiv M, Ben-Sira D. Effects of aerobic exercise training on S-Klotho in young and elderly. *J J Physiol* 2015; **1**: 1–2.

50 Mostafidi E, Moeen A, Nasri H, Ghorbani Hagjo A, Ardalan M. Serum Klotho Levels in Trained Athletes. *Nephrourol Mon* 2016; **8**: e30245.

51 Santos-Dias A, MacKenzie B, Oliveira-Junior MC, Moyses RM, Consolim-Colombo FM, Vieira RP. Longevity protein klotho is induced by a single bout of exercise. *Br J Sports Med* 2017; **51**: 549–550.

52 Saghiv M, Sagiv M, Ben-Sira D. Comparison between aerobic and anaerobic training influence on s-klotho blood levels following 60 min aerobic bout. *J J Physiol* 2015; **1**: 1–4.

53 Matsubara T, Miyaki A, Akazawa N. Aerobic exercise training increases plasma Klotho levels and reduces arterial stiffness in postmenopausal women. *Am J Physiol Hear Circ Physiol* 2014; **306**: 348–355.

54 Kurl S, Laukkanen JA, Rauramaa R, Lakka TA, Sivenius J, Salonen JT. Cardiorespiratory Fitness and the Risk for Stroke in Men. *Arch Intern Med* 2003; **163**: 1682.

55 Myers J, McAuley P, Lavie C, Despres J, Arena R, Kokkinos P. Physical activity and cardiorespiratory fitness as major markers of cardiovascular risk: their independent and interwoven importance to health status. *Prog Cardiovasc Dis* 2015; **57**: 306–314.

56 Nelson ME, Rejeski WJ, Blair SN, Duncan PW, Judge JO, King AC *et al.* Physical Activity and Public Health in Older Adults. *Med Sci Sport Exerc* 2007; **39**: 1435–1445.

57 Semba R, Cappola A, Sun K. Relationship of low plasma klotho with poor grip strength in older community-dwelling adults: the InCHIANTI study. *Eur J Appl Physiol* 2012; **112**: 1215–1220.

58 Shardell M, Semba RD, Kalyani RR, Hicks GE, Bandinelli S, Ferrucci L. Serum 25-Hydroxyvitamin D, Plasma Klotho, and Lower-Extremity Physical Performance Among Older Adults: Findings From the

InCHIANTI Study. *J Gerontol A Biol Sci Med Sci* 2015; **70**: 1156–62.

59 Baldan A, Giusti A, Bosi C, Malaventura C, Musso M, Forni GL *et al.* Klotho, a new marker for osteoporosis and muscle strength in β-thalassemia major. *Blood Cells Mol Dis* 2015; **55**: 396–401.

60 Semba RD, Ferrucci L, Sun K, Simonsick E, Turner R, Miljkovic I *et al.* Low Plasma Klotho Concentrations and Decline of Knee Strength in Older Adults. *J Gerontol A Biol Sci Med Sci* 2016; **71**: 103–8.

61 Guralnik J, Simonsick E. A short physical performance battery assessing lower extremity function: association with self-reported disability and prediction of mortality and nursing home admission. *J Gerontol* 1998; **49**: 85–94.

AIMS & HYPOTHESIS

AIMS

The overall aim of this International Doctoral Thesis is to study the effect of different exercise training programs on S-Klotho, as well as in physical fitness, energy metabolism, and cardiometabolic health, and to study the role of S-Klotho on physical fitness, energy metabolism and cardiometabolic health in sedentary middle-aged adults. This overall aim is addressed in ten studies. In addition, this International Doctoral Thesis also contains six methodological studies that were conducted to solve some methodological aspects to be applied in the rest of studies.

GENERAL INTRODUCTION

General objective: To study the available evidence on the associations between exercise and S-Klotho protein regulation **(Study 1)**.

METHODOLOGICAL SECTION

General methodological objective 1: To describe rationale, design and methodology of the FIT-AGEING randomized controlled trial **(Study 2)**.

General methodological objective 2: To determine the best methods for data collection, selection and analysis for assessing energy metabolism (i.e. MFO and REE) in healthy humans.

Specific methodological objective 2.1: To systematically review the available studies describing and/or comparing different data collection and analysis approach factors that could affect MFO in healthy individuals **(Study 3)**.

Specific methodological objective 2.2: To investigate the impact of using a pre-defined time interval on MFO, as wells as the impact of applying 2 different data analysis approaches (measured-values vs. polynomial-curve) on MFO estimations in sedentary adults **(Study 4)**.

Specific methodological objective 2.3: To study the RER at which MFO occurred in sedentary and trained healthy adults **(Study 5)**.

Specific methodological objective 2.4: To analyze the diurnal variation of MFO in trained male athletes **(Study 6)**.

Specific methodological objective 2.5: To provide normative values by sex, weight status, and age for MFO in sedentary healthy individuals evaluated by a treadmill test **(Study 7)**.

Specific methodological objective 2.6: To determine the accuracy and validity of REE predictive equations in normal-weight, overweight and obese sedentary middle-aged adults **(Study 8)**.

SECTION 1: S-Klotho protein and physical fitness

General objective 1: To examine the association of body composition and physical fitness with S-Klotho in sedentary middle-aged adults.

Specific objective 1.1: To analyse the association of body composition including LM and FM as well as BMD with S-Klotho in sedentary middle-aged adults **(Study 9)**.

Specific objective 1.2: To determine the association of sedentary, physical activity, and physical fitness levels (i.e. cardiorespiratory fitness and muscular strength) with S-Klotho in sedentary middle-aged adults **(Study 10)**.

SECTION 2: S-Klotho protein, energy metabolism and cardiometabolic health

General objective 2: To study the relationship of energy metabolism, cardiometabolic health and S-Klotho in sedentary middle-aged adults.

Specific objective 2.1: To examine the association of BMR and fuel oxidation in basal conditions and during exercise with S-Klotho in sedentary middle-aged adults **(Study 11)**.

Specific objective 2.2: To investigate the association of cardiometabolic risk with S-Klotho in sedentary middle-aged adults **(Study 12)**.

SECTION 3: Role of exercise on S-Klotho protein, physical fitness, energy metabolism and cardiometabolic health

General objective 3: To study the effects of different exercise training modalities on S-Klotho, physical fitness, energy metabolism, and cardiometabolic health in sedentary middle-aged adults.

Specific objective 3.1: To examine the effects of different exercise training modalities on S-Klotho in sedentary middle-aged adults **(Study 13)**.

Specific objective 3.2: To investigate the effects of different exercise training modalities on body composition in sedentary middle-aged adults **(Study 14)**.

Specific objective 3.3: To describe the influence of different exercise training modalities on physical fitness in sedentary middle-aged adults **(Study 15)**.

Specific objective 3.4: To investigate the influence of different exercise training programs on BMR and fat oxidation, in basal conditions and during exercise in sedentary middle-aged adults **(Study 16)**.

Specific objective 3.5: To describe the influence of different exercise training modalities on cardiometabolic risk in sedentary middle-aged adults **(Study 17)**.

HYPOTHESIS

The overall hypothesis of this International Doctoral Thesis is that S-Klotho is associated with physical fitness, energy metabolism and cardiometabolic health in sedentary middle-aged adults, and that exercise training, specially a HIIT+EMS program, increases S-Klotho, as well as physical fitness, energy metabolism, and cardiometabolic health.

SECTION 1: S-Klotho protein and physical fitness

General hypothesis 1: Body composition and physical fitness are associated with S-Klotho in sedentary middle-aged adults.

Specific hypothesis 1.1: LM and BMD are positively associated with S-Klotho and FM is negatively associated with S Klotho in sedentary middle-aged adults **(Study 9)**.

Specific hypothesis 1.2: Physical activity and physical fitness levels (i.e. cardiorespiratory fitness and muscular strength) are positively associated with S-Klotho, and sedentary time is negatively associated with S-Klotho in sedentary middle-aged adults **(Study 10)**.

SECTION 2: S-Klotho protein, energy metabolism and cardiometabolic health

General hypothesis 2: Energy metabolism and cardiometabolic health are positively associated with S-Klotho in sedentary middle-aged adults.

Specific hypothesis 2.1: BMR, BFox and MFO are positively associated with S-Klotho, whereas BCHox is negatively associated with S-Klotho in sedentary middle-aged adults **(Study 11)**.

Specific hypothesis 2.2: An increased cardiometabolic risk will be associated with poorer S-Klotho in sedentary middle-aged adults **(Study 12)**.

SECTION 3: Role of exercise on S-Klotho protein, physical fitness, energy metabolism and cardiometabolic health

General hypothesis 3: Exercise training, specially a HIIT+EMS program, increases S-Klotho, as well as physical fitness, energy metabolism, and cardiometabolic health in sedentary middle-aged adults.

Specific hypothesis 3.1: Exercise training, specially a HIIT+EMS program, increases S-Klotho in sedentary middle-aged adults **(Study 13)**.

Specific hypothesis 3.2: Exercise training, specially a HIIT+EMS program, improves body composition in sedentary middle-aged adults **(Study 14)**.

Specific hypothesis 3.3: Exercise training, specially a HIIT+EMS program, increases physical fitness in sedentary middle-aged adults **(Study 15)**.

Specific hypothesis 3.4: Exercise training, specially a HIIT+EMS program, increases BMR and fat oxidation, in basal conditions and during exercise in sedentary middle-aged adults **(Study 16)**.

Specific hypothesis 3.5: Exercise training, specially a HIIT+EMS program, decreases cardiometabolic risk in sedentary middle-aged adults **(Study 17)**.

MATERIAL & METHODS

This section has two chapters:

(i) Chapter 3 describes the rationale, design, and methodology of the FIT-AGEING study focusing on the periodization of PAR, HIIT and HIIT-EMS training programs, as well as its dependent outcomes (**Study 2**).

(ii) Chapter 4 includes a number of methodological studies investigating the measurement of BMR and MFO. Firstly, we performed a systematic review regarding the assessment of MFO **(Study 3)**. After that, we conducted 3 studies [i] to investigate the impact of using a pre-defined time interval and different data analysis approaches on MFO **(Study 4)**; [ii] to study the RER at which MFO occurred **(Study 5)**; and to analyze the diurnal variation of MFO **(Study 6)**. In addition, normative values by sex, weight status, and age for MFO are provided in **Study 7**. Finally, the accuracy and validity of REE predictive equations in normal-weight, overweight and obese sedentary middle-aged adults was examined in **Study 8**.

Chapter 3:
Exercise training as S-Klotho protein stimulator in sedentary healthy adults: Rationale, design, and methodology
(Study 2)

ABSTRACT

The secreted form of the a-Klotho gene (S-Klotho), which is considered a powerful biomarker of longevity, makes it an attractive target as an anti-ageing therapy against functional decline, sarcopenic obesity, metabolic and cardiovascular diseases, osteoporosis, and neurodegenerative disorders. The S-Klotho plasma levels could be related to physical exercise inasmuch physical exercise is involved in physiological pathways that regulate the S-Klotho plasma levels. FIT-AGEING will determine the effect of different training modalities on the S-Klotho plasma levels (primary outcome) in sedentary healthy adults. FIT-AGEING will also investigate the physiological consequences of activating the klotho gene (secondary outcomes).

FIT-AGEING will recruit 80 sedentary, healthy adults (50% women) aged 45- 65 years old. Eligible participants will be randomly assigned to a non-exercise group, i.e. the control group, (n=20), a physical activity recommendation from World Health Organization group (n=20), a high intensity interval training group (n=20), and a whole-body electromyostimulation group (n=20). The laboratory measurements will be taken at the baseline and 12 weeks later including the S-Klotho plasma levels, physical fitness (cardiorespiratory fitness, muscular strength), body composition, basal metabolic rate, heart rate variability, maximal fat oxidation, health blood biomarkers, free-living physical activity, sleep habits, reaction time, cognitive variables, and health-related questionnaires. We will also obtain dietary habits data and cardiovascular disease risk factors.

DESIGN

The present study is a randomized controlled trial (ClinicalTrials.gov ID: NCT03334357) approved by The Human Research Ethics Committee of the "*Junta de Andalucía*" [0838-N-2017]. All participants had to provide an informed consent. The participants were randomly allocated to a control group, a PAR group, (3) a HIIT group, and (4) a HIIT+EMS group. All of the baseline and follow-up examinations were performed at the same setting [*Centro de Investigación Deporte y Salud (CIDS)* at the University of Granada]. The study followed the revised ethical guidelines of the Declaration of Helsinki.

PARTICIPANTS AND SELECTION CRITERIA

The participants were adults from the province of Granada (Spain). Granada has ≈885,000 population, of which ≈190,000 are adults aged 45-65. The eligible participants were 45-65 years old, and they had a BMI between 18.5 to 35 kg/m^2. The inclusion and exclusion criteria are listed in Table 1. We decided to conduct the intervention on overweight and obese adults because overweight and obesity accelerate the ageing of adipose tissue, increase the formation of reactive oxygen species in fat cells, shorten telomeres, and produce the inhibition of the p53 tumor suppressor [1], which are factors that could be related to S-Klotho. Considering that aerobic exercise is able to increase S-Klotho in normal-weight young and senior adults according to literature [2], we also includes participants with a BMI between 18.5 and 24.9 kg/m^2. Including people with different weight status and body composition allows to study S-Klotho differences across different BMI categories (i.e. normal-weight, overweight, and obese based on BMI, and on body fat measures).

All participants had a health history and a medical examination done prior to the intervention program to minimize risks by ruling out contraindications to the testing and training protocols. If any participant suffered any injury or medical problem, a medical evaluation was performed and, if necessary, they were excluded from the study.

The study was announced on social networks, local media, and posters at different points of Granada. We also organized information meetings at the School of Medicine of the University of Granada. The people interested contacted the research team by e-mail and phone. Later, they visited our facilities to receive a thorough explanation about the study's aims, the test to be performed, the inclusion and exclusion criteria, and the types of intervention. The potentially interested participants meeting the inclusion criteria were invited to a second orientation session; in this case, the participants received detailed written information about the study, and the informed consent. The participants were cited for their baseline measurement.

Table 1. Selection criteria

Inclusion criteria	Exclusion criteria
Age: 45-65 years old	History of cardiovascular disease
BMI: 18.5-35 kg/m^2	Diabetes
Not engaged in regular physical activity >20min on >3days/week	Pregnancy or planning to get pregnant during the study period
Not participating in a weight-loss program	Beta blockers or benzodiazepines use
Stable weight over the last 5 months (weight changes>5kg)	Taking medication for thyroid
The participants must be capable and willing to provide consent, understand the exclusion criteria, and accept the randomized group assignment	Other significant conditions that are life-threatening or that can interfere with or be aggravated by exercise
Normal electrocardiogram	Unwillingness to either complete the study requirements or to be randomized into the control or training group

RANDOMIZATION AND BLINDING

After completing the baseline measurements, the selected participants were randomly assigned to either the control or the exercise training groups. The randomization was computer-generated using simple randomization [3]. The assessment staff was blinded to the participant randomization assignment. The participants were explicitly informed of their assigned group, as well as of the study hypotheses. They were frequently reminded not to disclose their randomization assignments to the assessment staff in the follow-up measurements. For practical and feasibility reasons, the study was conducted in two waves (maximum 45 participants).

SAMPLE SIZE

The determination of the sample size and power of the study were made based on the data of a pilot S-Klotho samples [4]. We considered S-Klotho differences between pre and post-treatment in order to assess the sample size requirements for the one-way ANOVA [5]. As a result, we expected to detect an effect size of 100 pg/ml considering a type I error of 0.05 with a statistical power of 0.85 if we recruited a minimum of 14 participants per group. Assuming a maximum loss at follow-up of 25%, we decided to recruit at least 20 participants (≈50% women) for each study group: control, PAR, HIIT, and HIIT+EMS. A total of 80 participants were planned to be enrolled in FIT-AGEING study. We used IBM-SPSS Sample power software (version 3.0.1) for calculations.

PARTICIPANT RETENTION AND ADHERENCE

The participants were allowed to withdraw at any time; however, to reduce participants drop-out and to maintain adherence to the training program, several strategies were implemented. In anticipation of private commitments, vacations, etc., the intervention program was carried out from September to December. All sessions were accompanied by music, and were held on an airy, well-lighted, and well-equipped gym. Qualified and certificated trainers were carefully supervised every training session, and they worked with groups of no more than six persons to ensure that the participants did the exercises correctly, and at a correct intensity. The training specialist and other study staff constantly supported the participants.

EXERCISE PROGRAM RATIONALE

Since there is no information regarding the ideal exercise model to induce higher levels of S-Klotho, the FIT-AGEING study applied different exercise training modalities. These methods were (i) PAR [6–9], (ii) HIIT, and (iii) HIIT+EMS.

One of the most important aims of the current randomized controlled trial was to compare various exercise intensity levels (moderate vs. high intensity) to test if higher intensity levels provide more benefits despite the application of a lower training volume.

The trial length were twelve weeks based on (i) the results of a previous study [10] and (ii) taking into account that the substantial physiological adaptations occur within the first 12-24 weeks of exercise [6]. We provided no dietary prescriptions or instructions to the participants in the control and exercise groups. The participants were asked to maintain their dietary habits during the intervention period.

PAR training program

Volume

Given the importance of the transferability of results to the general population in terms of time, intensity, and frequency, the volume of PAR were based on the minimum physical activity recommended by the World Health Organization (150min/week at moderate intensity) [11,12].

Intensity

Physical activity at moderate intensity is recommended to sedentary people by important health institutions to obtain health benefits [7,8,13]. Physical activity at 60% of the HRres produces significant physiological adaptations in sedentary adults [7,8,13]. For this reason, the intensity selected for PAR aerobic training was 60-65% of the HRres. 1RM is the maximum amount of force that can be generated in one maximal contraction, and it is used to determine the intensity of resistance training [14]. The World Health Organization recommends an intensity of 40-50% of 1RM to improve muscle strength, and to increase muscle mass in sedentary persons which have never done strength training [9]. Therefore, the intensity selected for this training modality was 40-50% of 1RM. We also considered other variables that influence strength training, such as eccentric-isometric-concentric speed ratio, recovery time, and range of motion [15].

Frequency

Considering several studies that have compared the effects of different training frequencies on physical fitness as a health marker, the World Health Organization recommends a dose of 3 or 4 days/week [13]. Because the lack of time is one of the most important causes of participant dropout, we determined that the PAR group trained 3 days/week, the minimum frequency recommended. Resistance training was performed on 2 of these 3 days/week. In addition, the participants were advised to refrain from training for a minimum of 24 hours and ideally 48 hours. The participants were phoned if they did not meet the weekly recommendations.

Type of exercise.

The ergometers selected for the aerobic training section were treadmill, cycle-ergometer, and elliptical ergometer. For the resistance training section, we included weight-bearing and guided pneumatic machines, involving the major upper and lower body muscle groups [9].

Training load variation

We considered that participants may not be immediately capable of meeting the volume and intensity dose required. Therefore, we proposed a gradual progression to control the exercise dose (see Table 2) based on a previous randomized controlled trial [16]. The participants started their training program with an aerobic dose of 75 min/week at 60% of the HRres. It was progressive increased 30 min/week, and the participants achieved 150 min/week on the 4th week. Regarding resistance training, the participants performed a 2-week familiarization phase; they learnt the movement patterns which are based on resistance exercises of our specific training program (dead lift, squat, horizontal, and vertical push-pull, etc.). In addition, the participants did compensatory exercises to improve core competency and joints stabilization, in order to avoid injuries. It is well known that, as the fitness level of participants increases, aerobic and strength load should be higher. Aerobic training intensity was increased, since it was necessary to rise the previously established intensity to maintain a specific percentage of the HRres, when the physical fitness was increased. In addition, we measured the 1RM of all exercises on the first week of each phase in order to adjust the resistance training load.

Table 2. PAR training periodization

		AEROBIC TRAINING		RESISTANCE TRAINING			
PHASES	**WEEKS**	**Volume (min)**	**Intensity (%HRres)**	**Intensity (%RM)**	**Type of exercise**	**Training stimulus**	
FAMILIARIZATION	**WEEK 1**	75	60	*Weight-bearing and elastic band*	*Movement pattern and global movements*	*Movement pattern*	*Compensatory training*
	WEEK 2	105	60				
	WEEK 3	120	60		**1RM ASSESTMENT**		
PHASE I	**WEEK 4**	150	60	50	*Exercises involving major muscle groups*	*Initial adaptations to resistance training*	*Compensatory training*
	WEEK 5	150	60	50			
	WEEK 6	150	60	50			
	WEEK 7	150	60	50			
	WEEK 8	120	60		**1RM ASSESTMENT**		
PHASE II	**WEEK 9**	150	60	50	*Exercises involving major muscle groups*	***Session type A**: mechanical tension and muscle damage*	***Session type B**: Metabolic Stress*
	WEEK 10	150	60	50			
	WEEK 11	150	60	50			
	WEEK 12	150	60	50			

PAR: Physical Activity Recommendations for adults proposed by the World Health Organization, HRres: Heart Rate Reserve, 1RM: Repetition Maximum

On the other hand, it is important to consider that the session organization determines different physiological adaptations in terms of muscle hypertrophy (metabolic stress, muscle damage etc.) [15]. Due to the fact that the best training stimulus to induce higher levels of the S-Klotho is unknown, we included different types of sessions during each training phase.

Training periodization

The training periodization is shown in Table 2. It was divided into two different phases, and its duration was of 5 weeks, starting with a familiarization phase. The training program structure was based on other randomized controlled trials, which had the aim to meet the physical activity recommendations for adults suggested by World Health Organization [7,16].

Familiarization phase: This phase was extended for two weeks. The principal aim of this phase was to learn the main movement patterns (squat, hinge, bridge, and horizontal and vertical pulls and push) and to improve many physical fitness components such as cardiorespiratory fitness, core stability, joint stabilizing muscles, balance, and flexibility. These sessions prepared the participants for the 1RM evaluation.

Phase I: The participants performed two combined sessions (aerobic and resistance training) and only one aerobic training session in phase 1. The aerobic training volume was 150 min/week (except in RM weeks, with a duration of 120 min/week) and the aerobic training intensity was 60% of the HRres in all cases. The resistance training included exercises involving the major muscle groups and principal movement patterns (squat, bench press, dead lift, lateral pull down…) and compensatory exercises. Combined sessions had type I structure (see Table 3) which alternate 4 resistance exercises involving the major muscle groups, 2 core stability exercises, and 2 compensatory exercises with 10-minute sets of aerobic training.

Phase II: In this case, the combined sessions were different in order to provide a different resistance training stimulus [7,15,17]. The combined session was divided into type I session (which focuses on mechanical tension and muscle damage) and type II session (which focuses on metabolic stress) [15,17]. Both sessions included similar exercises to those reported in phase 1, as well as several exercises which involve small muscle groups (lateral raises, French press, or lateral raises).

Training sessions

The participants completed a total of 60 minutes of aerobic exercise in non-combined sessions (only aerobic exercises). These sessions started with a dynamic standardized warm-up, including several muscle activation exercises. In addition, aerobic sessions included compensatory exercises. All combined training sessions began like a non-combined session.

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				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				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 VI	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: Physical Activity Recommendations for adults proposed by the World Health Organization, HRres: Heart Rate Reserve, RM: Repetition Maximum, Reps: Repetitions, Min: minutes.

After the warm-up, an aerobic exercise was carried out on 10-minute sets, alternating with resistance exercises (depending on the session [see Table 3]). The participants had the possibility to change the ergometer in different 10-minute aerobic sets (treadmill, elliptical, or cycle-ergometer). In all cases, the training session ended with a cooling-down protocol (active global stretching); the participants completed 5 anterior or posterior chain exercises.

HIIT program

HIIT describes physical exercise characterized by short and intermittent efforts of vigorous activity, interspersed with resting periods at passive or low-intensity exercises. There are many HIIT protocols, and the specific physiological adaptations induced by this training modality are related to exercise stimulus, (i.e. the intensity, duration, or number of intervals performed), as well as the duration and activity patterns during recovery [18,19]. The energy expenditure at HIIT (more intensity and low volume) is the same (or even higher in some cases) as moderate intensity exercise. However, HIIT physiological and health-related markers are better in healthy and diseased populations [19-21]. These findings are important from a public health perspective, because the 'lack of time' is one of the most common problems to do exercise.

Volume

The volume in HIIT (40-65 min/week at high intensity) was smaller than the minimum physical activity recommended by the World Health Organization (75 min/week at vigorous intensity).

Intensity

The HIIT intensity is based on scientific evidence [18,19,22,23]. HIIT participants performed two different complementary protocols: (i) HIIT with long intervals (Type A session), with an intensity of >95% of VO_2max and (ii) HIIT with short intervals (Type B session), with an intensity of >120% of VO_2max (>90% of the HRres or <9 {0-10 RPE scale} [24]). The intensity was progressively increased after the familiarization phase.

Frequency

Traditionally, HIIT has been recommended 3 times/week [22,23]. However, considering the age of the participants (45-65 years old) and their training level (sedentary), we decided to reduce the training frequency (twice per week), since this population needs a 72-hour rest after a HIIT session [25].

Type of exercise

The exercises programmed for HIIT with long intervals (type A session) were walking on a treadmill with personalized slopes. For the HIIT with short intervals (type B session), the participants performed eight weight-bearing exercises in circuit form, (i.e. squat, dead lift, high knees up, high heels up, push up, horizontal row, lateral plank, and frontal plank).

Training load variation

We considered that participants were not immediately capable of meeting the volume and intensity dose required; therefore, we proposed a gradual progression to control the exercise dose. The participant started with a dose of <40 min/week at 80%-90% of VO_2max in type A and type B sessions (HIIT familiarization phase). It was progressive increased to 50 min/week at >95% in type A session and 120% of VO_2max in type B session (HIIT phase I) and to 65 min/week at >95% in type A session and 120% of VO_2max in type B session (HIIT phase II).

Training periodization

The training periodization can be seen in Table 4. It is divided into three phases: (1) HIIT familiarization phase, (2) HIIT phase I, (3) HIIT phase II.

HIIT familiarization phase: This phase was extended for 4 weeks. The participants carried out 2 types of sessions each week, type A and type B. The intensity selected for the first and the second week was 80% of VO_2max. The intensity and the volume were higher in the third and fourth week. In session type A, the participants completed 6-9 sets of 4 minutes (2 minutes work/2 minutes rest) with a maximal duration of 18 minutes/session. In session type B, the participants completed 2 sets (8-9.5 minutes) of 16 exercises (15-20 seconds work / 15-20 seconds rest) with an active rest of 5 minutes at 60% of VO_2max and a maximal duration of 19 minutes/session.

HIIT Phase I: The participants did two different sessions as in the familiarization phase. The intensity was >95%VO_2max in type A session, and >120%VO_2max in type B session. The training volume was less than 50 minutes/week. In session type A, the participants completed 8-10 sets of 4 minutes (2 minutes work / 2 minutes rest) with a maximal duration of 20 minutes/session. In session type B, the participants completed 2 sets (8-12.5 minutes of duration) of 16 exercises (15-30 seconds work / 15-30 seconds rest) with an active rest of 5 minutes at 60% of VO_2max and a maximal duration of 25 minutes/session.

HIIT Phase II: Sessions followed the same structure than *HIIT Phase I*. However, training volume was more than 50 minutes/week but less than 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 (intensity >95% of VO_2max). In session type B, the participants completed 3 sets (8-12.5 minutes duration) of 16 exercises (15-30 seconds work / 15-30 seconds rest) with an active rest of 5 minutes to 60% of VO_2max and a maximal duration of 37 minutes/session (intensity >120% of VO_2max). The exercises in session type B were modified in order to increase their difficulty (add external load, increase range of motion, add instability, etc.) because it was expected that physical fitness increases as a training adaptation.

Training sessions

Type A session: It started with a dynamic standardized warm-up, including several muscle activation exercises followed by 5 minutes of aerobic exercise on the treadmill at 60% of VO_2max. After the warm-up, the participants completed several treadmill sets following the established parameters previously described.

Type B session: It started with a dynamic standardized warm-up. The participants performed eight weight-bearing exercises (in circuit form) twice per set with an active rest (walking at 60%VO_2max) following the periodization previously established.

In all cases, the training session ended with the same cooling-down protocol described in PAR.

HIIT+EMS program

WB-EMS is becoming increasingly popular as a novel training technology. WB-EMS is able to simultaneously stimulate up to 14–18 regions or 8–12 different muscle groups with up to 2.800 cm^2 electrode area [26].

Table 4. HIIT training periodization

HIIT familiarization phase

Week	**1**		**2**		**3**		**4**	
Session (type)	1 (A)	2 (B)	3 (A)	4 (B)	5 (A)	6 (B)	7 (A)	8 (B)
Exercises	1 (Tr)	8x2 = 16 (W-B)	1 (Tr)	8x2 = 16 (W-B)	1 (Tr)	8x2 = 16 (W-B)	1 (Tr)	8x2 = 16 (W-B)
Volume	12 min	16 min	14 min	21 min	16 min	16 min	18 min	21 min
Intensity	80% VO2m	80% VO2m	80% VO2m	80% VO2m	90% VO2m	90% VO2m	90% VO2m	90% VO2m
Sets	6	2	7	2	8	2	9	2
Set duration	4 min	8 min	4 min	10.5 min	4 min	8 min	4 min	10.5 min
Work exercise	2 min	15 Sec	2 min	20 Sec	2 min	15 Sec	2 min	20 Sec
Rest exercise	2 min (pass)	15 Sec	2 min (pass)	20 Sec	2 min (pass)	15 Sec	2 min (pass)	20 Sec
Rest between sets	-	5 min (60% VO2m)	-	5 min (60% VO2m)	-	5 min (60% VO2m)	-	5 min (60% VO2m)

HIIT Phase I

Week	**5**		**6**		**7**		**8**	
Session (type)	9 (A)	10 (B)	11 (A)	12 (B)	13 (A)	14 (B)	15 (A)	16 (B)
Exercises	1 (Tr)	8x2 = 16 (W-B)	1 (Tr)	8x2 = 16 (W-B)	1 (Tr)	8x2 = 16 (W-B)	1 (Tr)	8x2 = 16 (W-B)
Volume	16 min	16 min	18 min	21 min	20 min	27 min	20 min	32 min
Intensity	>95% VO2m	120% VO2m	>95% VO2m	120% VO2m	>95% VO2m	120% VO2m	>95% VO2m	120% VO2m
Sets	8	2	9	2	10	2	10	2
Set duration	4 min	8 min	4 min	10.5 min	4 min	13.5 min	4 min	16 min
Work exercise	2 min	15 Sec	2 min	20 Sec	2 min	25 Sec	2 min	30 Sec
Rest exercise	2 min (pass)	15 Sec	2 min (pass)	20 Sec	2 min (pass)	25 Sec	2 min (pass)	30 Sec
Rest between sets	-	5 min (60% VO2m)	-	5 min (60% VO2m)	-	5 min (60% VO2m)	-	5 min (60% VO2m)

HIIT Phase II

Week	**9**		**10**		**11**		**12**	
Session (type)	17 (A)	18 (B)	19 (A)	20 (B)	21 (A)	22 (B)	23 (A)	24 (B)
Exercises	1 (Tr)	8x2 = 16 (W-B)	1 (Tr)	8x2 = 16 (W-B)	1 (Tr)	8x2 = 16 (W-B)	1 (Tr)	8x2 = 16 (W-B)
Volume	18 min	24 min	21 min	31.5 min	24 min	40.5 min	24 min	40.5 min
Intensity	>95% VO2m	120% VO2m	>95% VO2m	120% VO2m	>95% VO2m	120% VO2m	>95% VO2m	120% VO2m
Sets	6	3	7	3	8	3	8	3
Set duration	4 min	8 min	4 min	10.5 min	4 min	13.5 min	4 min	13.5 min
Work exercise	3 min	15 Sec	3 min	20 Sec	3 min	25 Sec	3 min	25 Sec
Rest exercise	2 min (pass)	15 Sec	2 min (pass)	20 Sec	2 min (pass)	25 Sec	2 min (pass)	25 Sec
Rest between sets	-	5 min (60% VO2m)	-	5 min (60% VO2m)	-	5 min (60% VO2m)	-	5 min (60% VO2m)

Type A; High Intensity Interval Training on the treadmill with individual slopes, Type B; High Intensity Interval Power Training (weight-bearing exercises), Tr; Treadmill, W-B; Weight-Bearing exercises, VO_2m; Maximal oxygen uptake, Min; minutes, Sec; seconds, Pas; Passive.

Very few studies have determined the influence of WB-EMS on ageing, physical fitness, body composition, and physiological parameters in sedentary healthy adults [27–29], and its effects are controversial [30,31]. It is essential to follow the scientific recommendations related to WB-EMS to avoid possible health problems [32,33] produced by the irresponsible use of this technology [28,29,33]. The HIIT+EMS program followed the same structure as the HIIT intervention in terms of volume, intensity, frequency, type of exercise, training load variation, training periodization, and training session. However, electrical impulses were included in order to assess whether the WB-EMS stimuli produces an extra effect compared with the HIIT program.

Electrical parameters

Given that the participants have never done WB-EMS, we decided to establish a progressive and gradual HIIT-EMS training periodization in order to avoid possible dangerous health consequences, such as increased creatine kinase levels and rhabdomyolysis [30,31]. Using the WB-EMS devices from Wiemspro® (Malaga, Spain), bipolar, symmetrical, and rectangular electric pulse were applied. The periodization of electric parameters can be seen in Table 5. The typical frequency used in WB-EMS studies has been 85 Hz [26–29,33]. However, our participants were sedentary adults aged 45-65, and it has been shown that ageing is associated with a muscle mass decrease, especially type II fibers, and this decrease in muscle tissue begins around the age of 50 and dramatically increases beyond the age of 60 [34,35]. In addition, it is well-known that the ideal frequency to recruit type I fibers is 7-33 Hz [36]. Therefore, we applied a frequency of 15-33 Hz in our type A session (aerobic exercise). On the other hand, we applied a frequency of 35-75 Hz in the type B session (resistance exercises) because, in this case, our aim was to active type II fibers and their optimal frequency is 35-100 Hz [36]. The intensity applied in our intervention program was 80-100 mA, following the scientific guidelines established in local electrostimulation in order to improve fitness and body composition (>50 mA) [36]. The impulse intensity was individually adapted in accordance with the participants in order to generate similar values of RPE than other WB-EMS studies (49, 54-59) using the Borg CR-10 Scale "5" of "9" [37]. The scientific recommendations regarding this matter range between 200-400 µsec. We adjusted this parameter in relation to the body segment: thigh zone (400µsec), glute zone (350µsec), abdominal zone (300µsec), dorsal zone (250µsec), cervical (200µsec), chest zone (200µsec), and arm zone (200µsec) [36]. The stimulation ratio (duty cycle) is defined as ratio of on-time to the total cycle time (% duty cycle = 100/ [total time/on-time]). A duty cycle of 50-70% was used [27–29,38].

Table 5. Electrical parameters in the HIIT+EMS periodization.

FAMILIARIZATION PHASE								
Week	**1**		**2**		**3**		**4**	
Session (type)	1 (A)	2 (B)	3 (A)	4 (B)	5 (A)	6 (B)	7 (A)	8 (B)
Frequency	15 Hz	35 Hz	15 Hz	35 Hz	15 Hz	40 Hz	15 Hz	40 Hz
Intensity	100 mA	80 mA	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	7-8	7-8
Duty cycle	99% (59'':1'')	50% (15'':15'')	99% (59'':1'')	57% (20'':15'')	99% (59'':1'')	50% (15'':15'')	99% (59'':1'')	57% (20'':15'')
HIIT PHASE I								
Week	**5**		**6**		**7**		**8**	
Session (type)	9 (A)	10 (B)	11 (A)	12 (B)	13 (A)	14 (B)	15 (A)	16 (B)
Frequency	20 Hz	45 Hz	20 Hz	45 Hz	20 Hz	50 Hz	20 Hz	55 Hz
Intensity	100 mA	80 mA	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	7-8	7-8
Duty cycle	99% (59'':1'')	50% (15'':15'')	99% (59'':1'')	57% (20'':15'')	99% (59'':1'')	63% (25'':15'')	99% (59'':1'')	67% (30'':15'')
HIIT PHASE II								
Week	**9**		**10**		**11**		**12**	
Session (type)	17 (A)	18 (B)	19 (A)	20 (B)	21 (A)	22 (B)	23 (A)	24 (B)
Frequency	25 Hz	60 Hz	20 Hz	65 Hz	20 Hz	70 Hz	20 Hz	75 Hz
Intensity	100 mA	80 mA	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	8-9	8-9
Duty cycle	99% (59'':1'')	50% (15'':15'')	99% (59'':1'')	57% (20'':15'')	99% (59'':1'')	63% (25'':15'')	99% (59'':1'')	63% (25'':15'')

Type A; High Intensity Interval Training on the treadmill with individual slopes, Type B; High Intensity Interval P Training (weight-bearing exercises), Hz; Hertz, mA; milliamps.

We programmed a duty cycle of 50-67% in type B (resistance training) session following scientific evidence, but duty cycle in type A session (aerobic exercise) was 99% because the frequency was low and the work time was of 3 minutes maximum.

CONTROL GROUP

We provided general advice to the control group participants though an information meeting presided by a graduate in Sport Sciences. They were instructed to maintain their lifestyle.

OUTCOME VARIABLES

The primary outcome of our study was S-Klotho. The secondary outcome variables included physical fitness components, body composition and anthropometric measurements, energy expenditure and fuel oxidation in basal conditions and during exercise, health blood biomarkers, sedentary and physical activity levels, dietary habits, and cardiovascular risk factors (secondary outcomes) in sedentary healthy adults.

The baseline measurements were organized on 4 days:

Day 1: Medical examination (anamnesis, blood pressure assessment…) and fasting blood sample collection.

Day 2: BMR and fuel oxidation measured by IC during 30 minutes, body composition by Dual Energy X-ray Absorptiometry scan and anthropometric measurements, and MFO during an incremental treadmill protocol. All tests were conducted under fasting conditions.

Day 3: Upper and lower muscular strength by an isokinetic dynamometry test, manual isometric dynamometry and core resistance stability.

Day 4: Cardiorespiratory fitness by a maximum exercise test on a treadmill (H/P/Cosmos Pulsar, H/P/Cosmos Sport & Medical GMBH, Germany) with an Ultima CardiO2 metabolic cart (Medgraphics Corp, Minnesota, USA), electrocardiogram, and blood pressure control. All tests were supervised by a graduate in sport sciences, and a sport medicine doctor.

We used accelerometers to objectively measure sedentary and physical activity levels. Finally, we controlled the dietary intake by three 24 h recalls.

Primary outcome: S-Klotho

We collected blood samples from the antecubital vein after 12 hours of fasting. S-Klotho plasma levels were measured by ELISA using a soluble α-klotho ELISA assay kit (Demeditec, Kiel, Germany). The kit is a non-competitive solid-phase sandwich ELISA that uses two types of highly specific antibodies (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. All participants were requested to abstain from drugs and/or caffeine, to eat a standardized dinner before sampling, and to avoid any physical activity of moderate intensity (24 hours before) and/or vigorous intensity (48 hours before).

We also measured other blood parameters including a general biochemical profile and a hormone profile. The ELISA kits and spectrophotometry were used to perform these analyses.

Secondary outcomes

Physical fitness

Cardiorespiratory fitness were measured through a maximum treadmill test applying the modified Balke protocol [39], which has been widely used and validated [16,40–42]. We also measured the O_2 uptake and CO_2 production with a breath by breath gas analyzer calibrated with known gas mixtures and environmental air immediately before the test. Consistently across each trial, the participants were strongly encouraged to invest maximum effort. The criteria for achieving VO_2max were to reach a RER ≥1.1, a plateau in VO_2 (change of <100 ml/min in the last 30 s stage), and a heart rate within 10 beats/min of the age-predicted maximal heart rate (208–0.7∗age) [43]. The exercise electrocardiogram was continuously monitored.

We conducted an isokinetic strength test using a Gymnex Iso-2 dynamometer (EASYTECH s.r.l., Italy), calibrated following the product instructions before the data collection. The knee flexor and extensor muscles were tested concentrically and eccentrically at 180° and 60° s-1. The upper members, hips, and shoulders were stabilized with safety belts. The rotational axis of the dynamometer was aligned with the right lateral femoral condyle. The force pad was placed 3–4 cm above the medial malleolus. The knee extension started with a 90º-joint angle and it ended at 170°. The subjects were instructed to sub maximally flex and extend the knee five times, and then completed three maximal repetitions. We allowed the participants a one-minute rest between submaximal and maximal trials, and 5 minutes between 180° and 60° s-1, following a scientific validated protocol [44]. The peak torque was determined as the single repetition with the highest muscular force output (Nm). The participants were encouraged by the trainer during the test, and the same trainer-researcher conducted all the isokinetic tests.

We measured the handgrip strength using a digital dynamometer (TKK 5101 Grip-D; Takey, Tokyo, Japan). It was assessed following the procedures described elsewhere [45].

We evaluated the core resistance stability using a standard protocol described by McGill et al. [46], which has been extensively used in scientific studies [47,48]. This methodology included the Biering-Sorensen extensor endurance test, flexor endurance test (60º), frontal plank test, and the side bridge test.

Anthropometric and body composition measurements

Weight, height, hip circumference, and WC were determined following the recommended standardization procedures from the International Society for the Advancement of Kinanthropometry. We also evaluated the FM, FFM, LM, VAT, and BMD by conducting a dual-energy X-ray absorptiometer scan (Discovery Wi, Hologic, Inc., Bedford, MA, USA).

Energy expenditure and fuel oxidation

We evaluated the BMR and fuel oxidation by IC with a breath by breath gas analyzer. The participants were requested to attend to our lab in post-absorptive conditions (12–14 h fasting), to be abstained from drugs and/or caffeine, to eat an established dinner before blood samples, to avoid physical activity of moderate intensity (24 hours before) and/or vigorous intensity (48 hours before). They lied on a bed, in a quiet environment. Indirect calorimetric measures followed the scientific accepted standards to ensure the validity of these tests [49,50].

A standardized treadmill protocol test was used to measure MFO [51]. The test started at 3.5 $km \cdot h^{-1}$ and at a gradient of 0% during three min. The speed was then increased until reaching the maximal speed that the participant would comfortably maintain without running. Grade was increased by 2% every 3 min until a RER of 1 was reached. After that, the speed was decreased until 4 km/h, and the grade was 0% during 5 minutes (active recovery). The respiratory gas measurements were continuously monitorized. Furthermore, the heart rate and RPE record were measured throughout the whole test.

Sedentary and physical activity levels

The amount of sedentary and physical activity time was measured by accelerometry (Actigraph, Pensacola, Florida, USA). The participants wore two accelerometers (non-dominant wrist and right hip) during 7 consecutive days for 24 hours.

Dietary assessment

We conducted a dietary assessment based on three 24-hour dietary recalls (1 during the weekend) at the baseline and after the intervention. All data were processed by the dietetic software (EVALFINUT®, Ibero-American Foundation of Nutrition, Spain).

STUDIES´ METHODOLOGY OVERVIEW

The present International Doctoral thesis contains a total of a total of 17 studies. One of them (Study 1) was conducted to study the available evidence on the associations between exercise and S-Klotho protein regulation. Seven of them were methodological studies: one aimed to describe the rationale, design and methodology of the FIT-AGEING randomized controlled trial (Study 2), and the other six studies were conducted to determine the best methods for data collection, selection and analysis for assessing energy metabolism (i.e. MFO and REE) in healthy humans (Studies 3 to 8). The rest of studies were conducted to address the aims of the International Doctoral Thesis. All studies contain data from the participants enrolled in the FIT-AGEING project, except: (i) in study 4, 5, and 6 in which the participants of the ACTIBATE project (ClinicalTrials.gov ID: NCT02365129) were also included, and (ii) in study 6 in which an independent cohort of trained male athletes was considered. An overview of the design, cohorts, and variables included in every study is included below.

Study	General aim	Design	Cohort and participants	Study outcomes
Study 1	To study the available evidence on the associations between exercise and S-Klotho protein regulation	Systematic review	-	S-Klotho
Study 2	To describe rationale, design and methodology of the FIT-AGEING randomized controlled trial	Descriptive	FIT-AGEING (N=80)	S-Klotho Cardiometabolic risk factors Physical fitness BMR/BFox/MFO
Study 3	To systematically review the available studies describing and/or comparing different data collection and analysis approach factors that could affect MFO in healthy individuals	Systematic review	-	MFO
Study 4	To investigate the impact of using a pre-defined time interval on MFO, as wells as the impact of applying 2 different data analysis approaches (measured-values vs. polynomial-curve) on MFO estimations in sedentary adults	Cross-sectional	FIT-AGEING (N=42) and ACTIBATE (N=109)	MFO

Study 5	To study the RER at which MFO occurred in sedentary and trained healthy adults	Descriptive	FIT-AGEING (N=42) and ACTIBATE (N=125)	MFO
Study 6	To analyze the diurnal variation of MFO in trained male athletes	Repeated measured	Trained male athletes (N=12)	MFO VO_2max VT2
Study 7	To provide normative values by sex, weight status, and age for MFO in sedentary healthy individuals evaluated by a treadmill test	Descriptive	FIT-AGEING (N=42) and ACTIBATE (N=125)	MFO
Study 8	To determine the accuracy and validity of REE predictive equations in normal-weight, overweight and obese sedentary middle-aged adults	Repeated measured	FIT-AGEING (N=73)	REE Body composition
Study 9	To analyse the association of body composition including LM and FM as well as BMD with S-Klotho in middle-aged sedentary adults	Cross-sectional	FIT-AGEING (N=74)	S-Klotho Body composition
Study 10	To determine the association of sedentary, physical activity, and physical fitness levels (i.e. cardiorespiratory fitness and muscular strength) with S-Klotho in middle-aged sedentary adults	Cross-sectional	FIT-AGEING (N=74)	S-Klotho Physical fitness Physical activity
Study 11	To examine the association of BMR and fuel oxidation in basal conditions and during exercise with S-Klotho in middle-aged sedentary adults	Cross-sectional	FIT-AGEING (N=74)	S-Klotho BMR BFox MFO

Study 12	To investigate the association of cardiometabolic risk with plasma S-Klotho in middle-aged sedentary adults	Cross-sectional	FIT-AGEING (N=74)	S-Klotho Cardiometabolic risk factors
Study 13	To examine the effects of different exercise training modalities on S-Klotho in sedentary middle-aged adults	Randomized controlled trial	FIT-AGEING (N=68)	S-Klotho
Study 14	To investigate the effects of different exercise training modalities on body composition in sedentary middle-aged adults	Randomized controlled trial	FIT-AGEING (N=65)	Body composition
Study 15	To describe the influence of different exercise training modalities on physical fitness in sedentary middle-aged adults	Randomized controlled trial	FIT-AGEING (N=74)	Physical fitness
Study 16	To investigate the influence of different exercise training programs on BMR and fat oxidation, in basal conditions and during exercise in sedentary middle-aged adults	Randomized controlled trial	FIT-AGEING (N=71)	BMR BFox MFO
Study 17	To describe the influence of different exercise training modalities on cardiometabolic risk in sedentary middle-aged adults	Randomized controlled trial	FIT-AGEING (N=71)	Cardiometabolic risk

REFERENCES

1 Ahima R. Connecting obesity, aging and diabetes. *Nat Med* 2009; **15**: 996–997.

2 Saghiv M, Goldhammer E, Sagiv M, Ben-Sira D. Effects of aerobic exercise training on S-Klotho in young and elderly. *J J Physiol* 2015; **1**: 1–2.

3 Schulz, KF, Grimes D. Generation of allocation sequences in randomised trials: chance, not choice. *Lancet* 2002; **359**: 515–519.

4 Hawkins D. *Identification of outliers*. Chapman an. London, 1980.

5 Chow, S, Wang, H, Shao J. *Sample size calculations in clinical research*. CRC. 2007.

6 Pedersen, BK, Saltin B. Evidence for prescribing exercise as therapy in chronic disease. *Scand J Med Sci Sport* 2006; **16**: 3–63.

7 WHO. Global recommendations on physical activity for health, World Health Organ publications. Geneva, Switzerland, 2015.

8 US. Department of Health and Human Services. Physical Activity Guidelines. 2016.

9 ACSM. American College of Sports Medicine position stand. Progression models in resistance training for healthy adults. *Med Sci Sport Exerc* 2009; **41**: 687–708.

10 Matsubara T, Miyaki A, Akazawa N, Choi Y, Ra S-G, Tanahashi K *et al.* Aerobic exercise training increases plasma Klotho levels and reduces arterial stiffness in postmenopausal women. *Am J Physiol Heart Circ Physiol* 2014; **306**: H348-55.

11 WHO | Global recommendations on physical activity for health. *WHO* 2015.

12 Piercy KL, Troiano RP, Ballard RM, Carlson SA, Fulton JE, Galuska DA *et al.* The physical activity guidelines for Americans. *JAMA - J Am Med Assoc* 2018; **320**: 2020–2028.

13 Garber, CE, Blissmer, B, Deschenes, M, Franklin, BA, Lamonte, MJ, Lee, IM, Swain D. American college of sports medicine position stand. Quantity and quality of exercise for developing and maintaining cardiorespiratory, musculoskeletal, and neuromotor fitness in apparently healthy adults: guidance for prescribing exercise. *Med SciSports Exerc* 2011; **43**: 1334–1359.

14 Marchese, AR, Hill A. *The essential guide to fitness: for the fitness instructor*. Pearson: Sydney (Australia), 2011.

15 Schoenfeld B. The mechanisms of muscle hypertrophy and their application to resistance training. *J Strength Cond Res* 2010; **24**: 2857–2872.

16 Sanchez-Delgado, G, Martinez-Tellez, B, Olza, J, Aguilera, CM, Labayen, I,... Ortega, FB, Ruiz J. Activating brown adipose tissue through exercise (ACTIBATE) in young adults: Rationale, design and methodology. *Contemp Clin Trials* 2015; **45**: 416–425.

17 Schoenfeld, BJ, Ogborn, D, Krieger J. Effects of resistance training frequency on measures of muscle hypertrophy: A

systematic review and meta-analysis. *Sport Med* 2016; **1**: 1–9.

18 Hwang, CL, Yoo, JK, Kim, HK, Hwang, MH, Handberg, EM, Petersen, JW, Christou D. Novel all-extremity high-intensity interval training improves aerobic fitness, cardiac function and insulin resistance in healthy older adults. *Exp Gerontol* 2016; **82**: 112–119.

19 Gibala, MJ, Little, JP, MacDonald, MJ, Hawley J. Physiological adaptations to low‐volume, high‐intensity interval training in health and disease. *J Physiol* 2012; **590**: 1077–1084.

20 Ulbrich, A, Angarten, VG, Netto, AS, Sties, SW, Bündchen, DC, de Mara, LS, de Carvalho T. Comparative effects of high intensity interval training versus moderate intensity continuous training on quality of life in patients with heart failure: Study protocol for a randomized controlled trial. *Clin Trials Regul Sci Cardiol* 2016; **13**: 21–28.

21 Hwang, CL, Chou, CH, Wu Y. Effect of aerobic interval training on exercise capacity and metabolic risk factors in people with cardiometabolic disorders: a meta-analysis. *J Cardiopulm Rehabil Prev* 2011; **31**.

22 Buchheit, M, Laursen P. High-intensity interval training, solutions to the programming puzzle. *Sport Med* 2013; **43**: 313–338.

23 Lunt, H, Draper, N, Marshall, HC, Logan, FJ, Hamlin, MJ, Shearman, JP, Frampton C. High intensity interval training in a real world setting: a randomized controlled feasibility study in overweight inactive adults, measuring change in maximal oxygen uptake. *PLoS One* 2014; **9**.

24 Borg GA. Psychophysical bases of perceived exertion. *Med Sci Sports Exerc* 1982; **14**: 377–81.

25 Herbert, P, Grace, FM, Sculthorpe N. Exercising caution: prolonged recovery to a single session of high intensity interval training in older men. *J Am Gerontol Soc* 2015; **1**: 13–19.

26 Filipovic A, Kleinöder H, Plück D, Hollmann W, Bloch W, Grau M. Influence of Whole-Body Electrostimulation on Human Red Blood Cell Deformability. *J Strength Cond Res* 2015; **29**: 2570–8.

27 Kemmler, Von-Stengel S, Schwarz J, Mayhew J. Effect of Whole-Body Electromyostimulation on Energy Expenditure During Exercise. *J Strength Cond Res* 2012; **26**: 240–245.

28 Kemmler, Schliffka R, Mayhew J, von Stengel S. Effects of whole-body electromyostimulation on resting metabolic rate, body composition, and maximum strength in postmenopausal women: The training and electrostimulation trial. *J Strength Cond Res* 2010; **24**: 1880–1887.

29 Kemmler, Teschler M, Weißenfels A, Bebenek M, Fröhlich M, Kohl M *et al.* Effects of Whole-Body Electromyostimulation versus High-Intensity Resistance Exercise on Body Composition and Strength: A

Randomized Controlled Study. *Complement Altern Med* 2016; **1**: 1–9.

30 Kemmler, Teschler M, Bebenek M, Von Stengel S. (Very) high Creatinkinase concentration after exertional whole-body electromyostimulation application: health risks and longitudinal adaptations. *Wiener Medizinische Wochenschrift* 2015; **165**: 427–435.

31 Finsterer J, Stöllberger C. Severe rhabdomyolysis after MIHA-bodytec® electrostimulation with previous mild hyper-CK-emia and non-compaction. *Int J Cardiol* 2015; **180**: 100–102.

32 Malnick, S, Band, Y, Alin, P, Maffiuletti N. It's time to regulate the use of whole body electrical stimulation. *BMJ* 2016; **352**: 1693.

33 Kemmler, Froehlich M, Stengel V, Kleinöder H. Whole-Body Electromyostimulation–The Need for Common Sense! Rationale and Guideline for a Safe and Effective Training. *Dtsch Z Sport* 2016; **67**.

34 Verdijk, LB, Koopman, R, Schaart, G, Meijer, K, Savelberg, HH, Loon L. Satellite cell content is specifically reduced in type II skeletal muscle fibers in the elderly. *Am J Physiol Metab* 2007; **292**: 151–157.

35 Deschenes M. Effects of aging on muscle fibre type and size. *Sport Med* 2004; **34**: 809–824.

36 Filipovic A, Kleinöder H, Dörmann U, Mester J. Electromyostimulation—a systematic review of the influence of training regimens and stimulation parameters on effectiveness in electromyostimulation training of selected strength parameters. *J Strength Cond Res* 2011; **25**: 3218–30.

37 Borg, E, Kaijser A. A comparison between three rating scales for perceived exertion and two different work tests. *Scand J Med Sci Sport* 2006; **16**: 57–69.

38 Von-Stengel S, Bebenek M, Engelke K, Kemmler W. Whole-Body Electromyostimulation to Fight Osteopenia in Elderly Females: The Randomized Controlled Training and Electrostimulation Trial (TEST-III). *J Osteoporosis* 2015; **11**: 1–7.

39 Balke, B, Ware R. An experimental study of physical fitness of Air Force personnel. *United States Armed Forces Med Journal* 1959; **10**: 675–688.

40 Wei, M, Kampert, JB, Barlow, CE, Nichaman, MZ, Gibbons, LW, Paffenbarger, RS, Blair S. Relationship between low cardiorespiratory fitness and mortality in normal-weight, overweight, and obese men. *Jama* 1999; **282**: 1547–1553.

41 Sui, X, LaMonte, MJ, Laditka, N, Hardin, JW, Chase, N, Hooker, SP, Blair S. Cardiorespiratory fitness and adiposity as mortality predictors in older adults. *J Am Med Assoc* 2007; **298**: 2507–2516.

42 Ortega, FB, Lee, DC, Katzmarzyk, T, Ruiz, JR, Sui, X, Church, TS, Blair S. The intriguing metabolically healthy but obese phenotype: cardiovascular prognosis and role of fitness. *Eur Heart J* 2013; **34**: 389–397.

43 Pallarés J, Morán-Navarro R.

Methodological approach to the cardiorespiratory endurance training. *J Sport Heal Res* 2012; **4**: 119–136.

44 Artero, EG, Espada-Fuentes, JC, Argüelles-Cienfuegos, J, Román, A, Gómez-López, PJ, Gutiérrez A. Effects of whole-body vibration and resistance training on knee extensors muscular performance. *Eur J Appl Physiol* 2012; **112**: 1371–1378.

45 Ruiz-Ruiz J, Mesa J, Gutiérrez A, Castillo M. Hand size influences optimal grip span in women but not in men. *J Hand Surg Am* 2002; **27**: 897–901.

46 McGill, S, Childs, A, Liebenson C. Endurance times for low back stabilization exercises: clinical targets for testing and training from a normal database. *Arch Phys Med Rehabil* 1999; **80**: 941–944.

47 Shamsi, MB, Rezaei, M, Zamanlou, M, Sadeghi, M, Pourahmadi M. Does core stability exercise improve lumbopelvic stability (through endurance tests) more than general exercise in chronic low back pain? A quasi-randomized controlled trial. *Physiother Theory Pract* 2016; **32**: 171–178.

48 Willson, J, Dougherty, CP, Ireland, ML, Davis I. Core stability and its relationship to lower extremity function and injury. *J Am Acad Orthop Surg* 2005; **13**: 316–325.

49 Fullmer, S, Benson-Davies, S, Earthman, CP, Frankenfield, DC, Gradwell, E, Lee, P, Trabulsi J. Evidence analysis library review of best practices for performing indirect calorimetry in healthy and Non-critically ill individuals. *J Acad Nutr Diet* 2015; **115**: 1417–1446.

50 Compher, C, Frankenfield, D, Keim, N, Roth-Yousey L. Best practice methods to apply to measurement of resting metabolic rate in adults: a systematic review. *J Am Diet Assoc* 2006; **106**: 881–903.

51 Achten J, Gleeson M, Jeukendrup AE. Determination of the exercise intensity that elicits maximal fat oxidation. *Med Sci Sports Exerc* 2002; **34**: 92–7.

Chapter 4:
Methodological considerations for energy metabolism assessment

Assessment of maximal fat oxidation: a systematic review ***(Study 3)***

ABSTRACT

MFO and Fat$_{max}$ are considered biological markers of metabolic health and performance. A wide range of studies have been performed to increase our knowledge about their regulation by exercise and/or nutritional intervention. However, numerous data collection and analysis approaches have been applied, which may have affected the MFO and Fat$_{max}$ estimation. We aimed to systematically review the available studies describing and/or comparing different data collection and analysis approach factors that could affect MFO and Fat$_{max}$ estimation in healthy individuals and patients. Two independent researchers performed the search. We included all original studies in which MFO and/or Fat$_{max}$ were estimated by indirect calorimetry through an incremental graded exercise protocol published from 2002 to 2019. This systematic review provides key information about the factors that could affect MFO and Fat$_{max}$ estimation: ergometer type, metabolic cart used, warm-up duration and intensity, stage duration and intensities imposed in the graded exercise protocol, time interval selected for data analysis, stoichiometric equation selected to estimate fat oxidation, data analysis approach, time of the day when the test was performed, fasting time/previous meal before the test, and testing days for MFO/Fat$_{max}$ and maximal oxygen uptake assessment. We suggest that researchers measuring MFO and Fat$_{max}$ should take into account these key methodological issues that can considerably affect the accuracy, validity, and reliability of the measurement. Likewise, when comparing different studies, it is important to check whether the above-mentioned key methodological issues are similar in such studies to avoid ambiguous and unacceptable comparisons.

BACKGROUND

The prevalence of overweight and obesity has dramatically increased over the last decades, being currently a worldwide public health problem [1,2]. Although obesity is multifactorial, its aetiology is mainly based on a chronic imbalance between energy intake and energy expenditure. This chronic imbalance is not always due to hyperphagia since a high proportion of overweight individuals present a low metabolic rate, low rates of fat oxidation, an impaired sympathetic nervous activity, and metabolic inflexibility [3].

Obese individuals present an impaired BFox [4]. However, less attention has been given to the study of fat oxidation during exercise [5], and the finding are so fat controversial. Several studies reported a lower MFO capacity during exercise and a lower Fat_{max} in individuals with obesity compared with normal-weight individuals [6–8]. However, recent studies have observed higher MFO and Fat_{max} in obese people compared with their lean counterparts [9]. Despite these apparently contradictory results, it seems clear that both MFO and Fat_{max} can be considered markers of metabolic health [7,10].

Several studies estimated fat oxidation over a range of exercise intensities and protocols [11,12] yet in some cases the exercise duration was too long (i.e. 15 minutes at six different workloads [12]) and the number of exercise intensities used to determine MFO and Fat_{max} were minimal arbitrarily selected (i.e. walking on a treadmill at 4.3 km/h at 0%, 3%, and 6% slope [11]). Since 2002, MFO and Fat_{max} have commonly been determined by IC through an incremental graded exercise protocol adapted to the population under study [8,13,14]. This allowed to improve the previous methodology used to estimate fat oxidation during exercise.

A recent systematic review confirmed a high variability in MFO and Fat_{max} across individuals with different biological characteristics [7], which can be attributed to a number of factors in addition to the weight status. These factors include the following: (i) Training status: trained endurance athletes have greater MFO than less-trained endurance athletes, with no differences in Fat_{max} [7,15,16]. (ii) Sex: absolute MFO (g/minute) is lower in women, whereas MFO relative to LM appears to be greater in women compared to men [7,17–19]. However, Fat_{max} seems to be higher in women than in men [7,18]. (iii) Nutritional status: a previous study compared MFO and Fat_{max} in two different conditions that included 75 g of glucose vs. placebo ingested 45 minutes pre-exercise after fasting overnight. They showed lower MFO and Fat_{max} in the glucose ingestion condition [18].

Besides the individual's biological characteristics [20], there are numerous factors that could affect MFO and Fat_{max} estimation related to data collection and analysis approach (i.e. ergometer type, metabolic cart used, warm-up duration and intensity, stage duration and intensities imposed in the graded exercise protocol, time interval selected for data analysis, stoichiometric equation selected to estimate fat oxidation, data analysis approach, time of the day when the test was performed, fasting time/previous meal before the test, and testing days for MFO/Fat_{max} and VO_2max assessment.

Numerous MFO and Fat_{max} data collection and analysis approaches have been previously applied, which could explain an important part of the high inter-individual variability and discrepant findings in the literature of MFO and Fat_{max} previously reported [20]. To our knowledge, there is currently no available systematic review focused on the different data collection and analysis approaches used in the MFO and Fat_{max} determination which allows to fully understand their role in the high inter-individual variability of MFO and Fat_{max}. In addition, there are no guidelines for the estimation of MFO and Fat_{max}.

Therefore, in order to provide specific recommendations for MFO and Fat_{max} estimation, we systematically reviewed the available studies describing and/or comparing different data collection and analysis approach factors that could affect the MFO and Fat_{max} estimation during an incremental graded exercise protocol in healthy individuals and patients.

MATERIAL & METHODS

Study design

This systematic review was registered in the International Prospective Register of Systematic Reviews (PROSPERO: identifier ID: 103158). The study was undertaken in accordance with the PRISMA statement [21].

The present review focuses on 10 key methodological issues related to MFO and Fat_{max} data collection and analysis approach: (i) ergometer type, (ii) metabolic cart used, (iii) warm-up protocol (duration and intensity), (iv) graded exercise protocol (stage duration and intensities imposed), (v) time interval selected for data analysis, (vi) stoichiometric equation selected to estimate fat oxidation, (vii) data analysis approach, (viii) time of the day when the test is performed, (ix) acute nutritional status (fasting time and previous meal), and (x) testing days for MFO/Fat_{max} and VO_2max assessment.

Search strategy

We searched in MEDLINE (via PubMed) and Web of Science for studies using incremental graded exercise protocols to measure MFO and Fat_{max}. The search was done using the Boolean search method, which limits the search results with operators including AND/OR/NOT to only those documents containing relevant key terms in the scope of this review. The search combined the following terms: "maximal fat oxidation", "fatmax", "peak fat oxidation", "fuel oxidation", "whole-body fat oxidation", "fat oxidation", "maximal lipid oxidation" "lipid oxidation", "exercise ", "training", "walking", "cycling", "running", "physical activity". The search equations were:

- PubMed:
    - (("maximal fat oxidation" or "fatmax" or "peak fat oxidation" or "fuel oxidation" or "whole-body fat oxidation" or "fat oxidation" or "maximal lipid oxidation" or "lipid oxidation")) AND ("exercise " or "training" or "walking" or "cycling" or "running" or "physical activity") NOT (((((((((((((((("Mice"[Mesh]) OR "Rats"[Mesh]) OR "Animal Experimentation"[Mesh]) OR "Models, Animal"[Mesh])) OR ("rats" OR "mouse"))) OR "mice")) OR "rat")))))))))
- Web of Science:
    - (((((((maximal fat oxid* or fatmax) or peak fat oxid*) or fuel oxid*) or whole-body fat oxid*) or fat oxid*) or maximal lipid oxid*) or lipid oxid*) AND (((((exercise or training) or walking) or cycling) or running) or physical activit*) NOT (Mice OR Rat* OR (Experiment* AND Animal*) OR (Research* AND Animal*) OR mouse OR (model* AND animal*).

Since the first experimental study that assessed MFO and Fat_{max} through incremental graded exercise protocol was published in 2002 [8], we limited the dates of our search from 1st January 2002 to 26th February 2019. When the MFO and Fat_{max} data collection and processing criteria were not specified in the manuscript, we contacted authors (3 manuscripts). Also, we carefully examined the reference lists of the selected studies as an additional verification for potential studies that could be included in this review (11 manuscripts).

Eligibility criteria

Research articles were selected using the defined PICOS (Population, Intervention, Comparison and Outcome) criteria [22], and the literature search only included original studies (cross-sectional, longitudinal, or follow-up) in which the MFO and Fat_{max} were measured by incremental graded exercise protocol. We did not find studies performed in individuals younger than 6 years old or older than 80 years old, consequently the studies included in this review concern individuals within 6 and 80 years old, which allowed us to classify the participants into older adults (aged >60), adults (aged 18-59), adolescents (aged 12-17), and children (aged 6-11). Reviews, editorials, and abstracts or congress communications were excluded. Studies were required to be written in English or Spanish language, and to be published in a peer-reviewed journal.

Data extraction

Two investigators (FAG and ADO) independently read the articles and checked whether they met the eligibility criteria, and a third reviewer was involved when discrepancies were found (LJF). Eighty-two percent of agreement was reached on selecting the papers in the first phase, and 100% of agreement after discrepancies was resolved in a consensus meeting. In addition to the 10 key methodological issues related to MFO and Fat_{max} data collection and analysis approach (see above), we extracted the following data from each study: (i) study (author identification and reference), (ii) number of participants and sex, (iii) participants' age, (iv) participants' health status and fitness level, (v) participants' weight status, and (vi) study design.

RESULTS

Figure 1 shows the PRISMA consort flow diagram of the search strategy and selection process. A total of 6915 manuscripts were identified, of which 315 were duplicates. Subsequently, we screened title and abstract of 6285 manuscripts, excluding 6147. Forty-one articles were additionally excluded after reading the full text and verifying that they did not meet the inclusion criteria. A total of 112 manuscripts were considered eligible for this systematic review. We identified a total of 2 studies (1.8%) conducted in older adults, 93 studies (83.0%) conducted in adults, 13 studies (11.6%) conducted in adolescents, and 9 studies (8.0%) conducted in children (Table 1). We identified a total of 85 cross-sectional studies (75.9%), 26 longitudinal studies (23.2%), and 1 follow-up study (0.9%). Figure 2 shows the percentage of studies that did not provide information about key methodological issues considered in the present systematic review.

Ergometer type

The ergometers used to assess MFO and Fat_{max} through an incremental graded exercise protocol were a cycle-ergometer (n=86 studies, 76.8%), a treadmill (n=32 studies, 28.6%), and a hand cycle ergometer (n=2 studies, 1.8%).

Metabolic cart used

A total of 14 metabolic carts were identified across the studies included (Table 2).

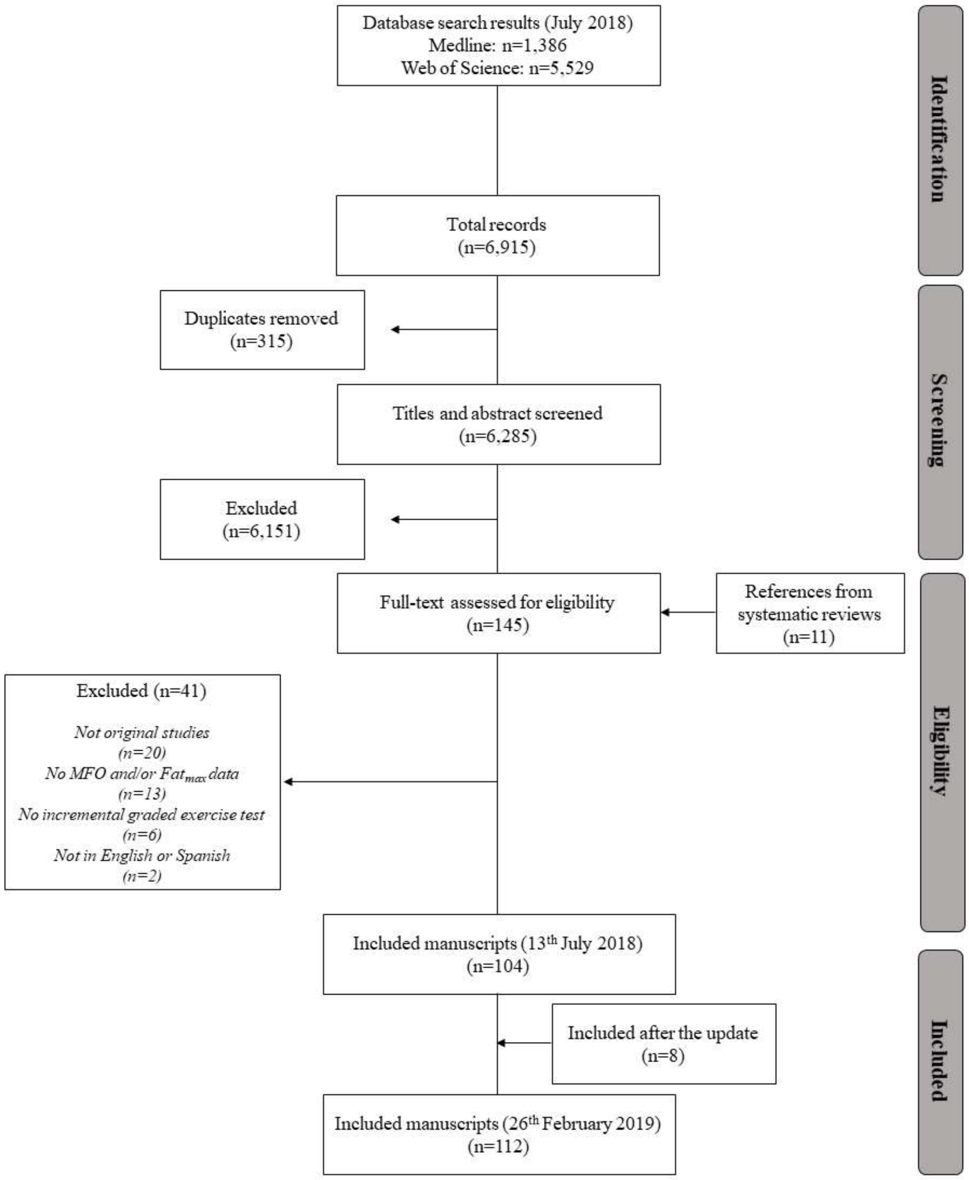

Figure 1. Flowchart of the literature search and study selection process. Abbreviations: MFO, maximal fat oxidation; Fatmax, exercise intensity eliciting MFO.

Table 1. Summary of the criteria used for data collection and data analysis in the articles reviewed.

Study	Participants (sex)	Fitness level (Health status)	Ergometer	Metabolic cart	Warm up protocol	Graded exercise protocol		Time interval	Stoichiometric equation	Data analysis approach	Time of the day	Acute nutritional status		Testing days for MFO/Fat$_{max}$ and VO_2max assessment
						Stage duration	*Intensity*					*Fasting time*	*Standard meal*	
Amaro-Gahete (2019)[1]	12 (men)	Trained (H)	Treadmill (walking)	CPX Ultima CardiO2	3 min at 3.5 km/h	3 min	Increments of 1 km/h of speed and 2% of grade	Last 60 sec	Frayn	3rd order polynomial curve	Morning - Afternoon	8 hours	Breakfast/ Lunch/ Dinner	1 day
Özgünen (2019)[2]	35 (men)	Sedentary (H)	Treadmill (walking)	Cosmed Quark	2 min at 3 km/h	6 min	Increments of 1 km/h	Last 60 sec	Frayn	Measured-values	Morning	12 hours	Not reported	2 days
Soria (2019)[3]	26 (men)	Trained (H)	Cyclo-ergometer	Oxycon Pro	10 min at 100 W	3 min	Increments of 30 W	Last 120 sec	Frayn	2nd order polynomial curve	Morning	Overnight	Breakfast/ Lunch/ Dinner	1 day
Astorino (2018)[4]	77 (men/women)	Active (H)	Cyclo-ergometer	TrueOne 2400	7 min at 40/30 W	3 min	Increments of 20 W	Last 60 sec	Frayn	Measured-values	Morning	Overnight	Not reported	1 day
Cancino-Ramírez (2018)[5]	60 (women)	Sedentary (H)	Cyclo-ergometer	Metalyzer 3B	3 min at 20% of Wt	6 min	30-40-50-60 of Wt	Last 120 sec	Frayn	Measured-values	Not reported	6 hours	Not reported	2 days
Chrzanowski-Smith (2018)[6]	16 (men/women)	Not reported (H)	Cyclo-ergometer	Mini MP 5200	Not reported	4 min	Increments of 25 W	Last 60 sec	Frayn	Measured-values	Morning	Overnight	Not reported	1 day
Cipryan (2018)[7]	18 (men)	Active (H)	Treadmill (running)	ZAN 600	4 min at 7.0 km/h	4 min	Increments of 1.5 km/h	Last 120 sec	Jeukendrup	Measured-values	Morning	3 hours	Not reported	1 day
Dandanell (2018)[8]	16 (men)	Untrained/trained (H)	Cyclo-ergometer	Oxycon Pro	3-5 min at 95 W	3 min	Increments of 25 W / 35W	Last 30 sec	Frayn	2nd order polynomial curve	Morning	Overnight	Breakfast/ Lunch/ Dinner	1 day

Desai (2018)[9]	11 (men/women)	Active (H)	Cyclo-ergometer	Metalyzer 3B	3 min at 30 W	3 min	Increments of 10 W	Last 60 sec	Jeukendrup	Measured-values	Morning	Overnight	Breakfast/ Lunch/ Dinner	1 day
Guadalupe-Grau (2018)[10]	11 (men/women)	Sedentary (Metabolic syndrome)	Cyclo-ergometer	Cosmed Quark	Not reported	Not reported	Not reported	Last 60 sec	Frayn	Not reported	Not reported	10 hours	Not reported	1 day
Gutierrez-Hellin (2018a)[11]	17 (men/women)	Moderately trained (H)	Cyclo-ergometer	Metalyzer 3B	10 min at 30% of VO_2max	3 min	40-50-60-70-80-90 % of VO_2max	Last 60 sec	Brouwer	Not reported	Morning	8 hours	Not reported	2 days
Gutierrez-Hellin (2018b)[12]	13 (men/women)	Active (H)	Cyclo-ergometer	Metalyzer 3B	10 min at 30% of VO_2max	3 min	40-50-60-70-80-90 % of VO_2max	Not reported	Brouwer	Measured-values	Morning	4 hours	Not reported	2 days
Larsen (2018)[13]	20 (men)	Sedentary (H/hypercholesterolemia)	Cyclo-ergometer	Oxycon Pro	6 min at 25 W	3 min	Increments of 25/20 W	Last 30 sec	Frayn	2nd order polynomial curve	Morning	Overnight	Not reported	2 days
Stein (2018)[14]	60 (women	Sedentary (Obesity)	Treadmill (walking)	CPX Ultima CardiO2	3 min at 3.5 km/h (1% grade)	3 min	Increments of 1km/h until 7.5 km/h. + increments of 2% grade	Last 120 sec	Frayn	Not reported	Morning	12 hours	Not reported	1 day
Tan (2018)[15]	34 (women)	Sedentary (T2DM)	Treadmill (walking)	Metalyzer 3B	3 min at 2.5 km/h (1% grade)	3 min	Increments of 0.5 km/h	Not reported	Frayn	Not reported	Morning	10 hours	Not reported	2 days

Vest (2018)[16]	38 (women)	Trained (H)	Cyclo-ergometer	Oxycon Pro	3 min at 60 W	3 min	Increments of 35 W	Last 90 sec	Frayn	2nd order polynomial curve	Morning	9-13 hours	Not reported	1 day
Yokoyama (2018)[17]	24 (men/women)	Not reported (H)	Cyclo-ergometer	Aero-monitor	3 min at 20 W	1 min	Increments of 20 W	Last 60 sec	Frayn	Measured-values	Not reported	3 hours	Not reported	2 days
Dandanell (2017a)[18]	80 (men/women)	Sedentary (Obesity)	Cyclo-ergometer	Cosmed Quark	6 min at 25 W	3 min	Increments of 25/20 W	Last 30 sec	Frayn	2nd order polynomial curve	Morning	12 hours	Not reported	1 day
Dandanell (2017b)[19]	16 (men/women)	Not reported (Obesity)	Cyclo-ergometer	Oxycon Pro	6 min at 30 W	3 min	Increments of 25/20 W	Last 30 sec	Frayn	3rd order polynomial curve	Morning	12 hours	Not reported	1 day
Durkalec-Michalski (2017)[20]	17 (men/women)	Trained (H)	Cyclo-ergometer	Cosmed Quark	Not reported	1.5 min	Increments of 25 W	Not reported	Jeukendrup	Not reported	Morning	Not reported	Not reported	1 day
Fletcher (2017)[21]	305 (men/women)	Not completely sedentary (H)	Treadmill (walking-running)	Oxycon Pro	3 min at 3.5 km/h	3 min	Increments of 1 km/h	Last 60 sec	Frayn	Measured-values	Morning	10-12 hours	Not reported	1 day
Frandsen (2017)[22]	64 (men)	Trained (H)	Cyclo-ergometer	Oxycon Pro	3 min at 60 W	3 min	Increments of 35 W	Last 90 sec	Frayn	3rd order polynomial curve	Morning	9-13 hours	Not reported	1 day
Morville (2017)[23]	6 (men)	Moderately trained (H)	Cyclo-ergometer	Cosmed Quark	5 min at 60 W	3 min	Increments of 35 W	Last 60 sec	Frayn	Measured-values	Morning	Overnight	Not reported	1 day

Peric (2017)[24]	57 (men)	Trained (H)	Treadmill (running)	Cosmed Quark	2 min at 6 km/h (1% grade)	2 min	Increments of 1 km/h	Not reported	Livesey	Measured-values	Morning	3 hours	Not reported	1 day
Ponce-González (2017)[25]	319 (men/women)	Moderately trained (H)	Cyclo-ergometer	Vmax N29	5 min at 30 W	3 min	Increments of 30 W	Last 60 sec	Frayn	Not reported	Morning	Overnight	Not reported	1 day
Randell (2017)[26]	1121 (men/women)	Trained (H)	Cyclo-ergometer	Moxus Modular	3 min at 5 km/h	3 min	Increments of 1 km/h	Between 90 sec to 150 sec	Brouwer	Measured-values	Morning	<5 hours	Not reported	1 day
Anderson-Hall (2016)[27]	12 (women)	Moderately trained (H)	Cyclo-ergometer	Oxycon Pro	5 min at 25% of VO_2max	3 min	30-40-50-60-70-80 % of VO_2max	Last 60 sec	Frayn	3rd order polynomial curve	Morning	Overnight	Dinner	1 day
Croci (2016)[28]	13 (men/women)	Sedentary (Non-alcoholic fatty liver disease)	Cyclo-ergometer	Not reported	5 min at 20% of VO_2max	5 min	Increments of 10% of VO_2max	Last 60 sec	Frayn	Not reported	Morning	10-12 hours	Non-standard	1 day
De Souza (2016)[29]	16 (men/women)	Moderately trained (H)	Treadmill (walking-running)	Metalyzer 3B	10 min at 70% of V_{Lc}	6 min	5 stages [(V_{Lc} + MAS) / 4]	Last 60 sec	Péronnet	3rd order polynomial curve	Morning	Overnight	Breakfast/ Lunch/ Dinner	2 days
Gutiérrez-Hellin (2016)[30]	18 (men)	Moderately trained (H)	Cyclo-ergometer	Metalyzer 3B	10 min at 50 W	3 min	Increments of 25 W	Last 60 sec	Frayn	Not reported	Morning	<8 hours	Not reported	1 day
Kim (2016)[31]	9 (men)	Sedentary (H)	Treadmill (running)	Aero-monitor	Not reported	3 min	Increments of 2% of grade and 1.5 mph	Not reported	Jeukendrup	Not reported	Morning - Afternoon	3 hours	Breakfast/ Lunch	1 day

Peric (2016)[32]	47 (men)	Sedentary/ trained (H)	Treadmill (walking-running)	Cosmed Quark	2 min at 6 km/h (1% grade)	2 min	Increments of 1km/h	Not reported	Livesey	Not reported	Morning	2 hours	Non-standard	1 day
Robinson (2016)[33]	57 (men)	Moderately trained (H)	Treadmill (walking-running)	Oxycon Pro	3 min at 3.5 km/h (1% grade)	3 min	Increments of 1km/h	Last 60 sec	Frayn	Measured-values	Morning	10 hours	Not reported	1 day
Tan (2016a)[34]	46 (men)	Sedentary (H)	Treadmill (walking)	Metalyzer 3B	3 min at 3 km/h (1% grade)	3 min	4 stages (4-5-6-6.5 km/h)	Last 60 sec	Frayn	Not reported	Morning	10 hours	Not reported	2 days
Tan (2016b)[35]	30 (women)	Sedentary (H)	Treadmill (walking)	Metalyzer 3B	3 min at 3.5 km/h (1% grade)	3 min	4 stages (4-5-6-6.5 km/h)	Last 60 sec	Frayn	Not reported	Morning	10 hours	Not reported	2 days
Alkhatib (2015)[36]	12 (men/women)	Active (H)	Cyclo-ergometer	Metalyzer 3B	Not reported	Not reported	Not reported	Last 30 sec	Péronnet	Measured-values	Morning	3-12 hours	Not reported	1 day
Besnier (2015)[37]	136 (women)	Sedentary (Obesity)	Cyclo-ergometer	Not reported	2 min at 15 W	5-6 min	20-30-40-50-60 % of MAS	Not reported	Péronnet	Not reported	Morning	Overnight	Not reported	2 days
Borel (2015)[38]	19 (women)	Sedentary (Metabolic syndrome)	Cyclo-ergometer	Ergocard	3 min at 20% of Wmax	6 min	30-40-50-60 % of Wmax	Last 60 sec	Péronnet	Measured-values	Morning	Overnight	Not reported	1 day
Guadalupe-Grau (2015)[39]	6 (men)	Not-reported (H)	Cyclo-ergometer	Oxycon Pro	5 min at 60 W	4 min	Increments of 35 W	Last 60 sec	Frayn	3rd order polynomial curve	Morning	10-12 hours	Not reported	2 days

Isacco (2015)[40]	21 (women)	Moderately trained (H)	Cyclo-ergometer	Oxycon Pro	6 min at 20% of Wmax	6 min	30-40-50-60 % of Wmax	Last 60 sec	Péronnet	Measured-values	Morning	Overnight	Not reported	1 day
Lanzi (2015)[41]	19 (men)	Sedentary/active (Obesity)	Cyclo-ergometer	Vmax N29	10 min at 20% of Wmax	5 min	Increments of 10% of Wmax	Last 60 sec	Frayn	2nd order polynomial curve	Morning	Overnight	Not reported	2 days
Marzouki (2015)[42]	12 (men)	Active (H)	Cyclo-ergometer + Treadmill	Cosmed Quark	Not reported	6 min	20-30-40-50-60 % of Wmax or MAS	Last 180 sec	Péronnet	Not reported	Morning	12 hours	Dinner	2 days
Mendelson (2015)[43]	40 (men/women)	Active (H) (H-Obesity)	Cyclo-ergometer	Ergocard	6 min at 20% of Wt	6 min	30-40-50-60 % of Wt	Last 60 sec	Péronnet	Not reported	Morning	Overnight	Not reported	2 days
Mohebbi (2015)[44]	9 (men)	Sedentary (H)	Cyclo-ergometer	Metalyzer 3B	3 min at 40 W	4 min	Increments of 20 W	Last 60 sec	Jeukendrup	Not reported	Morning	12 hours	Not reported	1 day
Nordby (2015)[45]	60 (men)	Moderately trained (H)	Cyclo-ergometer	Oxycon Pro	8 min at 60 W	4 min	Increments of 30 W	Last 90 sec	Frayn	Measured-values	Morning	12 hours	Not reported	2 days
Robinson (2015)[46]	57 (men)	Moderately trained (H)	Treadmill (walking-running)	Oxycon Pro	3 min at 3.5 km/h (1% grade	3 min	Increments of 1 km/h	Last 60 sec	Frayn	Measured-values	Morning	10 hours	Not reported	1 day
Rosenkilde (2015)[47]	50 (men)	Sedentary (H)	Cyclo-ergometer	Oxycon Pro	8 min at 20% of Wmax	3 min	30-40-50-60-70 % of Wmax	Last 90 sec	Frayn	Measured-values	Morning	Overnight	Not reported	2 days
Suk (2015)[48]	24 (women)	Not-reported (T2DM)	Cyclo-ergometer	Vmax N29	3 min at 20% of Wmax	3 min	30-40-50-60 % of Wmax	Not reported	Frayn	Not reported	Not reported	Not reported	Not reported	1 day

Wang (2015)[49]	30 (women)	Sedentary/active (H)	Treadmill (walking)	Metalyzer 3B	3 min at 3.5km/h (1% grade)	4 min	4 stages (4-5-6-6.5 km/h)	Not reported	Frayn	Not reported	Morning	10 hours	Not reported	2 days
Alkhatib (2014)[50]	14 (men/women)	Not-reported (H)	Cyclo-ergometer	Metalyzer 3B	Not reported	3 min	Increments of 0.5W/kg of body mass	Last 60 sec	Péronnet	Not reported	Morning	10 hours	Not reported	1 day
Alvehus (2014)[51]	17 (men)	Moderately trained (H)	Cyclo-ergometer	Oxycon Pro	3 min at 80 W	3 min	Increments of 40 W	Last 60 sec	Jeukendrup	Measured-values	Morning	1 hours	Breakfast	1 day
Astorino (2014)[52]	20 (men/women)	Sedentary/active (H)	Cyclo-ergometer	TrueOne 2400	4 min at 40 W	3 min	Increments of 20 W	Last 180 sec	Frayn	Not reported	Morning	3 hours / Overnight	Breakfast/ Lunch/ Dinner	1 day
Blaize (2014)[53]	12 (women)	Active (H)	Treadmill (walking-running)	TrueOne 2400	3 min at 3.5 km/h (1% grade)	3 min	Increments of 0.9 km/k until 9.3 km/h + increments of 2% grade	Last 120 sec	Frayn	Not reported	Not reported	4 hours	Not reported	2 days
Croci (2014a)[54]	24 (men)	Moderately trained (H)	Cyclo-ergometer	TrueOne 2400	5 min at 60 W	4 min	Increments of 30 W	Last 60 sec	Frayn	Sine model	Morning	10 hours	Not reported	2 days
Croci (2014b)[55]	15 (men)	Moderately trained (H)	Cyclo-ergometer	Oxycon Pro	6 min at 20% of Wmax	5 min	Increments of 7.5% of Wmax	Last 120 sec	Frayn	Measured-values + 3rd order polynomial curve + Sine model	Morning	12 hours	Not reported	1 day

Chu (2014)[56]	33 (women)	Not-reported (H)	Cyclo-ergometer	Vmax N29	4 min at 10 W	3 min	Increments of 10 W	Last 20 sec	Péronnet	2nd order polynomial curve	Morning	Overnight	Not reported	2 days
Isacco (2014)[57]	21 (women)	Not-reported (H)	Cyclo-ergometer	Oxycon Pro	6 min at 20% of Wt	6 min	Increments of 10 % of Wt	Last 60 sec	Péronnet	Measured-values	Morning	Not reported	Not reported	1 day
Jabbour (2014)[58]	37 (women)	Sedentary/ active (H/Obesity)	Cyclo-ergometer	Oxycon Pro	2 min at 25 W	2 min	Increments of 25 W	Last 30 sec	Péronnet	Not reported	Afternoon	4 hours	Breakfast/ Lunch	1 day
Lanzi (2014)[59]	32 (men)	Sedentary (H/Obesity)	Cyclo-ergometer	Vmax N29	10 min at 20% of Wmax	6 min	Increments of 7.5% of Wmax	Last 60 sec	Frayn	Sine model	Morning	12 hours	Not reported	2 days
Schwindling (2014)[60]	16 (men)	Trained (H)	Cyclo-ergometer	Metalyzer 3B	6 min at V_{Lc}	6 min	Increments of 30-50 W	Last 30 sec	Jeukendrup	Measured-values	Morning	Not reported	Breakfast/ Lunch/ Dinner	2 days
Takagi (2014)[61]	9 (men)	Moderately trained (H)	Treadmill (walking-running)	Aero-monitor	1 min at 1 km/h (0% grade)	1 min	Increments of 1 metabolic equivalent	Last 60 sec	Frayn	Measured-values	Morning	12 hours	Not reported	1 day
Astorino (2013)[62]	30 (women)	Sedentary (H)	Cyclo-ergometer	TrueOne 2400	4 min at 40 W	3 min	Increments of 20 W	Last 120 sec	Frayn	Not reported	Morning	12 hours	Not reported	1 day
Croci (2013)[63]	35 (men/women)	Not-reported (H/Non-alcoholic fatty liver disease)	Cyclo-ergometer	TrueOne 2400	5 min at 20% of Wt	5 min	Increments of 10% of Wt	Last 60 sec	Frayn	Measured-values	Morning	Overnight	Not reported	2 days

Decombaz (2013)[64]	22 (men)	Trained (H)	Cyclo-ergometer	Vmax N29	3 min at 60 W	4 min	Increments of 35 W	Not reported	Péronnet	2nd order polynomial curve	Morning	Overnight	Not reported	1 day
Chenevière (2012)[65]	15 (men)	Moderately trained (H)	Cyclo-ergometer	Oxycon Pro	10 min at 20% of Wmax	5 min	Increments of 7.5% of Wmax	Last 120 sec	Frayn	Sine model	Morning	10 hours	Not reported	2 days
Desplan (2012)[66]	60 (men)	Sedentary (Metabolic syndrome-T2DM)	Cyclo-ergometer	Oxycon Mobile	Not reported	6 min	20-30-40-50-60 % of Wmax	Last 180 sec	Jeukendrup	Not reported	Not reported	Not reported	Not reported	1 day
Gmada (2012)[67]	23 (men)	Sedentary (H)	Cyclo-ergometer	Cosmed Quark	3 min at 20% of Wmax	6 min	30-40-50-60 % of Wmax	Last 180 sec	Péronnet	2nd order polynomial curve	Morning	12 hours	Not reported	2 days
Makni (2012)[68]	131 (men/women)	Not-reported (Obesity)	Cyclo-ergometer	ZAN 600	6 min at 20% of Wmax	6 min	30-40-50-60 % of Wmax	Last 120 sec	Péronnet	Not reported	Morning	Not reported	Not reported	2 days
Mendelson (2012)[69]	15 (men/women)	Moderately trained (H)	Cyclo-ergometer + Treadmill	Metalyzer 3B	3 min at 20% of MAS	6 min	30-40-50-60 % of VO_2max	Last 120 sec	Péronnet	Measured-values	Morning	12 hours	Not reported	1 day
Tsujimoto (2012)[70]	15 (men)	Not-reported (Obesity)	Cyclo-ergometer	Aero-monitor	4 min at 15 W	4 min	Increments of 15 W	Last 90 sec	Péronnet	Not reported	Morning	Overnight	Dinner	2 days
Zakrzewski (2012a)[71]	27 (men/women)	Not-reported (H)	Treadmill (walking)	Metalyzer 3B	4 min at 4 - 4.5 km/h	4 min	Increments of 0.5 km/h	Last 60 sec	Frayn	2nd order polynomial curve	Morning	2 hours	Non-standard	2 days
Zakrzewski (2012b)[72]	25 (men/women)	Not-reported (H)	Cyclo-ergometer + Treadmill	Metalyzer 3B	3 min at 3 km/h or 0 W	3 min	Increments of 0.5 km/h or 6-8 W	Last 60 sec	Frayn	2nd order polynomial curve	Morning	2 hours	Breakfast/ Lunch/ Dinner	2 days

Ara (2011)[73]	30 (men)	Not-reported (Obesity)	Cyclo-ergometer / Hand ergometer	Oxycon Pro	5 min at 95 W / 20 W	5 min / 3 min	Increments of 35 W /15 W	Last 60 sec	Frayn	2nd order polynomial curve	Morning	Overnight	Not reported	1 day
Chenevière et. al (2011)[74]	24 (men/women)	Moderately trained (H)	Cyclo-ergometer	Oxycon Pro	10 min at 20% of Wmax	5 min	Increments of 7.5% of Wmax	Last 120 sec	Frayn	Sine model	Morning	10 hours	Not reported	2 days
Chu (2011)[75]	7 (men)	Not-reported (Obesity)	Cyclo-ergometer	Vmax N29	3 min at 12.5 W	3 min	Increments of 12.5 W	Last 30 sec	Frayn	Measured-values	Morning	Overnight	Not reported	2 days
González-Haro (2011)[76]	11 (men)	Trained (H)	Cyclo-ergometer	Cosmed Quark	10 min at 100 W	4 min	Increments of 30 W	Last 120 sec	Frayn	2nd order polynomial curve	Not reported	Not reported	Not reported	1 day
Mohebbi (2011)[77]	22 (men)	Sedentary (H)	Treadmill	Cosmed Quark	3 min at 3.5 km/h (1% grade)	3 min	Increments of 2% grade	Last 120 sec	Frayn	Measured-values	Morning / Evening	8-12 hours/ 5-6 hours	Not reported	1 day
Rynders (2011)[78]	148 (men/women)	Sedentary (H)	Cyclo-ergometer	Vmax N29	3 min at 40 W	3 min	Increments of 15 W	Last 60 sec	Frayn	Not reported	Not reported	4 hours	Non-standard	1 day
Zakrzewski (2011)[79]	30 (men/women)	Not-reported (H)	Cyclo-ergometer	K4 B2	3 min at 6-8 W	3 min	Increments of 6-8 W	Last 60 sec	Frayn	2nd order polynomial curve	Morning	12 hours	Not reported	2 days
Capostagno (2010)[80]	10 (men)	Trained (H)	Cyclo-ergometer + Treadmill	Oxycon Pro	10 min at 60 % of Wmax	10 min	Increments of 5% of Wmax	Not reported	Frayn	Not reported	Morning	Not reported	Breakfast/ Lunch/ Dinner	2 days
Chenevière (2010)[81]	13 (men/women)	Moderately trained (H)	Cyclo-ergometer + Treadmill	Oxycon Pro	5 min at 40 W or 3km/h	3 min	Increments of 20 W or 1 km/h	Last 60 sec	Frayn	Sine model	Not reported	<6 hours	Breakfast/ Lunch/ Dinner	1 day

Del Coso (2010)[82]	20 (men/women)	Trained/ Untrained (H)	Cyclo-ergometer	Cosmed Quark	Not reported	6 min	40-60-80 % of VO_2max	Last 180 sec	Brouwer	Not reported	Not reported	4 hours	Breakfast/ Lunch/ Dinner	2 days
Haufe (2010)[83]	129 (men/women)	Sedentary (Obesity)	Cyclo-ergometer	Vmax N29	2 min at 25 W	2 min	Increments of 25 W	Last 10 sec	Frayn	3rd order polynomial curve	Morning	2 hours	Breakfast	1 day
Lima-Silva (2010)[84]	18 (men)	Trained (H)	Treadmill (running)	K4 B2	3 min at 6 km/h	4 min	Increments of 1.2 km/h	Last 45 sec	Frayn	Measured-values	Not reported	Not reported	Not reported	1 day
Rosenkilde (2010)[85]	44 (men)	Sedentary (H)	Cyclo-ergometer	Oxycon Pro	8 min at 60 W	3 min	Increments of 30 W	Last 90 sec	Frayn	2nd order polynomial curve	Morning	Overnight	Not reported	1 day
Scharhag (2010)[86]	17 (men/women)	Sedentary/active (H)	Treadmill (running)	Metalyzer 3B	3 min at 4.5- 6 km/h	3 min	Increments of 1 km/h	Last 30 sec	Jeukendrup	Measured-values	Afternoon	<1 hour	Breakfast/ Lunch/ Dinner	2 days
Tolfrey (2010)[87]	19 (men/women)	Active (H)	Cyclo-ergometer	Servomex 1400 B4	8 min at 25-30 W	3 min	Increments of 10-12 W	Last 60 sec	Frayn	2nd order polynomial curve	Morning	11 hours	Dinner	2 days
Aucouturier (2009)[88]	24 (men/women)	Sedentary (Obesity)	Cyclo-ergometer	Oxycon Pro	4 min at 20% MAS	4 min	30-40-50-60 % of MAS	Last 60 sec	Péronnet	2nd order polynomial curve	Morning	Overnight	Not reported	1 day
Chenevière (2009a)[89]	20 (men/women)	Sedentary (H)	Treadmill (walking-running)	Oxycon Pro	5 min at 3 km/h (1% grade)	3 min	Increments of 1 km/h	Last 60 sec	Frayn	Sine model	Not reported	12 hours/ 6 hours	Breakfast/ Lunch/ Dinner	1 day
Chenevière (2009b)[90]	32 (men/women)	Moderately trained (H)	Cyclo-ergometer	Oxycon Pro	5 min at 40 W	3 min	Increments of 20 W	Last 60 sec	Frayn	Sine model	Morning - Afternoon	6 hours	Lunch	1 day
Larssen (2009)[91]	23 (men)	Not-reported (Obesity-T2DM)	Cyclo-ergometer / Hand ergometer	Oxycon Pro	5 min at 95 W / 20 W	5 min / 3 min	Increments of 35 W /15 W	Last 90 sec	Frayn	2nd order polynomial curve	Morning	Overnight	Not reported	1 day

Meyer (2009)[92]	21 (men/women)	Not-reported (H)	Cyclo-ergometer	Metalyzer 3B	Not reported	6 min	5 stages [(V_{Lc} + Wmax) / 4]	Last 30 sec	Péronnet	Measured-values	Not reported	3 hours	Breakfast/ Lunch/ Dinner	1 day
Mogensen (2009)[93]	27 (men)	Sedentary (T2DM)	Cyclo-ergometer	Oxycon Pro	4 min at 30 W	4 min	Increments of 30 W	Between 180 sec to 210 sec	Frayn	2nd order polynomial curve	Morning	Overnight	Not reported	2 days
Zunquin (2009a)[94]	46 (men)	Not-reported (Obesity)	Cyclo-ergometer	Ergocard	5 min at 0 W	3.5 min	Increments of 20 W	Last 30 sec	Péronnet	2nd order polynomial curve	Morning	2 hours	Breakfast	1 day
Zunquin (2009b)[95]	30 (men)	Not-reported (Obesity)	Cyclo-ergometer	Ergocard	5 min at 0 W	3.5 min	Increments of 20 W	Last 30 sec	Frayn	Measured-values	Morning	2 hours	Breakfast	1 day
Bogdanis (2008)[96]	46 (men/women)	Sedentary (H)	Treadmill (walking)	Vmax N29	5 min at 4-4.5 km/h	4 min	Increments of 0.3-0.5 km/h + increments of 1% grade	Last 60 sec	Jeukendrup	Measured-values	Not reported	4 hours	Not reported	1 day
Michalet (2008)[97]	14 (men/women)	Active (H)	Cyclo-ergometer	Ergocard	3 min at 20 % of Wt	6 min	30-40-50-60 % of Wt or Wmax	Last 120 sec	Péronnet	Not reported	Morning	12 hours	Not reported	1 day
Riddell (2008)[98]	15 (men/women)	Active (H)	Cyclo-ergometer	Vmax N29	3 min at 12.5 W	3 min	Increments of 12.5 W	Last 30 sec	Frayn	2nd order polynomial curve	Morning	Overnight	Not reported	1 day
Venables (2008)[99]	8 (men)	Sedentary/active (Obesity)	Treadmill (walking)	Oxycon Pro	3 min at 3.5 km/h (1% grade)	3 min	Increments of 1 km/h + increments of 2% grade	Last 120 sec	Jeukendrup	Not reported	Not reported	Not reported	Breakfast/ Lunch/ Dinner	1 day

González-Haro (2007)[100]	34 (men/women)	Trained (H)	Cyclo-ergometer	Cosmed Quark	10 min at 100 W	4 min	Increments of 30W	Last 120 sec	Frayn	Measured-values	Not reported	Not reported	Breakfast/ Lunch/ Dinner	1 day
Kang (2007)[101]	22 (men/women)	Active (H)	Cyclo-ergometer	Vmax N29	5 min at 25W	10 min	40-50-60-70 % of VO_2max	Last 120 sec	Not reported	Measured-values	Morning	4 hours	Breakfast/ Lunch/ Dinner	2 days
Bennard (2006)[102]	10 (men)	Moderately trained (H)	Cyclo-ergometer	Vmax N29	3 min at 95 W	5 min/ 3 min	Increments of 35W/ 20W	Not reported	Frayn	Not reported	Morning	Overnight	Not reported	2 days
Nordby (2006)[103]	16 (men)	Trained/ Untrained (H)	Cyclo-ergometer	Oxycon Pro	8 min at 60 W	3 min	Increments of 35 W	Last 90 sec	Frayn	2nd order polynomial curve	Morning	Overnight	Not reported	1 day
Stisen (2006)[104]	16 (women)	Trained/ Untrained (H)	Cyclo-ergometer	Oxycon Pro	15 min at 30% of VO_2max	3 min	Increments of 10-20 W	Last 60 sec	Frayn	3rd order polynomial curve	Morning	3 hours	Breakfast	2 days
Bircher (2005)[105]	78 (men/women)	Trained/ Moderately trained (H)	Cyclo-ergometer	Oxycon Pro	3 min at 60 W	3 min	Increments of 35 W	Last 120 sec	Frayn	Measured-values	Morning	10 hours	Dinner	1 day
Brandou (2005)[106]	14 (men/women)	Not-reported (Obesity)	Cyclo-ergometer	CPX Ultima CardiO2	6 min at 20% of Wt	6 min	30-40-50-60 % of Wt	Last 120 sec	Péronnet	Not reported	Not reported	12 hours	Not reported	2 days
Venables (2005)[107]	300 (men/women)	Not-reported (H)	Treadmill (walking)	Oxycon Pro	3 min at 3.5 km/h (1% grade)	3 min	Increments of 0.9 km/h + increments of 2% grade	Last 120 sec	Frayn	Measured-values	Not reported	4 hours	Not reported	1 day

Achten (2003a)[108]	11 (men)	Moderately trained (H)	Cyclo-ergometer	Oxycon Pro	3 min at 95 W	3 min	Increments of 35 W	Last 120 sec	Frayn	2nd order polynomial curve	Morning	Overnight/ 45min	Not reported	1 day
Achten (2003b)[109]	65 (men)	Moderately trained (H)	Cyclo-ergometer	Oxycon Pro	3 min at 95 W	3 min	Increments of 35 W	Last 120 sec	Frayn	2nd order polynomial curve	Morning	10-12 hours	Not reported	1 day
Achten (2003c)[110]	12 (men)	Moderately trained (H)	Cyclo-ergometer + Treadmill	Oxycon Pro	3 min at 95 W / 5.5 or 6.5 km/h	3 min	Increments of 35 W / increments of 2% grade	Last 120 sec	Frayn	2nd order polynomial curve	Morning	10-12 hours	Not reported	1 day
Brandou (2003)[111]	14 (men/women)	Not-reported (Obesity)	Cyclo-ergometer	CPX Ultima CardiO2	6 min at 20% of Wt	6 min	30-40-50-60 % of Wt	Last 120 sec	Péronnet	Not reported	Not reported	12 hours	Not reported	2 days
Achten (2002)[112]	12 (men)	Moderately trained (H)	Cyclo-ergometer	Oxycon Pro	3-5 min at 95 W	5 min/ 3 min	Increments of 35W/ 20W	Last 120 sec	Frayn	2nd order polynomial curve	Morning	10-12 hours	Not reported	1 day

Abbreviations: MFO; maximal fat oxidation, Fat_{max}; intensity that elicit MFO, VO_2max; maximal oxygen uptake; H; healthy, NW; normal-weight, OW: overweight, Min; minutes, Sec; seconds, VO_2max; maximal oxygen uptake, W; watios, Wt; Theoretical maximal load, T2DM; type II diabetes mellitus, V_{Lc}; velocities at which the aerobic threshold was reached, MAS; maximal aerobic speed, Mph; miles per hour, Wmax; maximum load (watios).

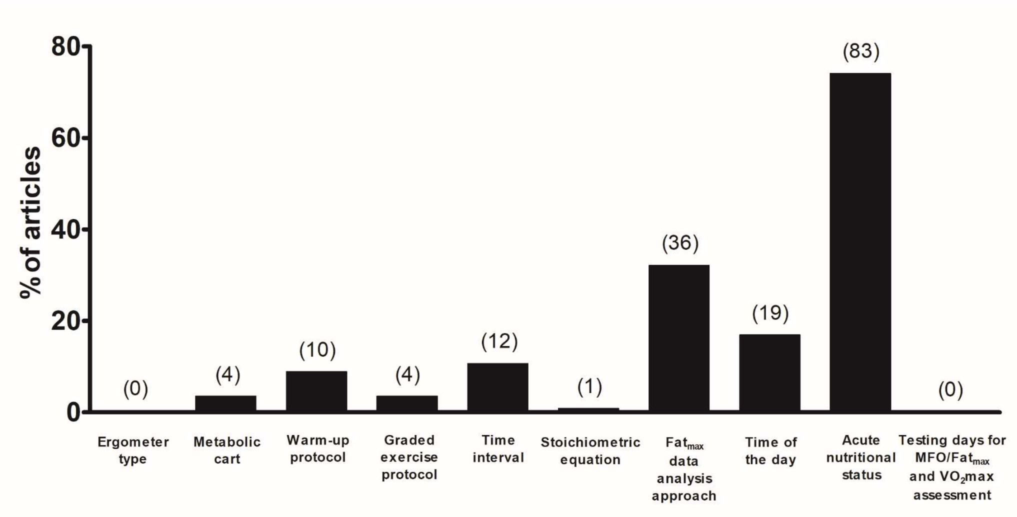

Figure 2. Percentage of the 112 included papers that did not report key methodological issues related to maximal fat oxidation (MFO) and the exercise intensity eliciting MFO (Fat_{max}) data collection and data analysis approach. Total number of studies that did not report information about the specific key methodological issue related to MFO and Fatmax data collection and data analysis approach are included in brackets.

Table 2. Metabolic carts identified across the studies included in the systematic review.

Metabolic cart	% of articles
Oxycon Pro (Jaeger, Hochberg, Germany)	33.0%
Metalyzer 3B (Cortex, Leipzig, Germany)	17.0%
Vmax N29 (Sensormedic, California, USA)	11.6%
Cosmed Quark (CPET, Rome, Italy)	11.6%
TrueOne 2400 (ParvoMedics, Sandy, UT)	5.3%
Ergocard (Schiller, Baar, Switzerland)	4.5%
Aeromonitor (Minato Medical Science, Tokyo, Japan)	3.6%
CPX Ultima CardiO2 (Medical Graphics Corp, St Paul, USA)	3.6%
K4 B2 (Cosmed, Rome, Italy)	1.8%
ZAN 600 (ZAN Messgeräte, Oberthulba, Germany)	1.8%
Moxus Modular (AEI, Technologies, Pittsburgh, USA)	0.9%
Mini MP 5200 (Servomex Group Ltd., Crowborough, East Sussex, UK)	0.9%
Oxycon Mobile (Viasys Healthcare GmbH, Hoechberg, Germany)	0.9%
Servomex 1400 B4 (East Sussex, UK)	0.9%

Warm-up protocol

We observed that the warm-up intensity was lower than the first load selected for the main part of incremental graded exercise protocol in all studies that provided information about the warm-up. The warm-up duration was (i) similar to the duration of their respective incremental graded exercise protocol stages in 50 studies (44.6%), (ii) higher than the duration of their respective incremental graded exercise protocol stages in 37 studies (33.0%), and (ii) lower than the duration of their respective incremental graded exercise protocol stages in 14 studies (12.5%).

Graded exercise protocol

There are a number of different stage durations during the graded exercise protocols across the studies selected: (i) 53 studies (47.3%) applied 3 minutes of stage duration, (ii) 19 studies (17.0%) applied 6 minutes of stage duration, (iii) 16 studies (14.3%) applied 4 minutes of stage duration, (iv) 11 studies (9.8%) applied 5 minutes of stage duration, (v) 4 studies (3.6%) applied 2 minutes of stage duration, (vi) 2 studies (1.8%) applied 10 minutes of stage duration, (vii) 2 studies (1.8%) applied 1 minute of stage duration, and (viii) 1 study (1.0%) applied 1.5 minutes of stage duration.

Time interval for data selection and analysis

The time interval selected for data analysis across the studies included were (i) the last 60 seconds of each stage in 44 studies (39.3%), (ii) the last 120 seconds of each stage in 25 studies (22.3%), (iii) the last 30 seconds of each stage in 13 studies (11.6%), (iv) the last 90 seconds of each stage in 7 studies (6.3%), (v) the last 45 seconds of each stage in 1 study (0.9%), (vi) the last 20 seconds of each stage in 1 study (0.9%), (vii) the last 10 seconds of each stage in 1 study (0.9%), (viii) between 90 to 150 seconds of each stage in 1 study (0.9%), and (ix) between 180 to 210 seconds of each stage in 1 study (0.9%).

Stoichiometric equation selected to estimate fat oxidation

We identified 5 stoichiometric equations to estimate fat oxidation: (i) the Frayn equation [125] in 72 studies (64.2%), (ii) the Péronnet equation [126] in 22 studies (19.6%), (iii) the Jeukendrup equation [127] in 11 studies (9.8%), (iv) the Brouwer equation [128] in 4 studies (3.6%), and (v) the Livesey equation [129] in 2 studies (1.8%).

Data analysis approach

The data analysis approaches used across the studies included were the following: (i) The measured-values data analysis approach was applied in 37 studies (33.0%). This data analysis approach is based on the highest fat oxidation rate recorded in the graded exercise protocol [75]. (ii) 25 studies applied the 2nd polynomial curve data analysis approach and 9 studies applied the 3rd polynomial data analysis approach (22.3% and 8.0%, respectively).

This data analysis approach is based on the graphical depiction of fat oxidation as a function of exercise intensity performing a 2nd or 3rd polynomial curve with intersection at (0;0). Fat_{max} can be calculated by differentiation of the 2nd or 3rd polynomial equation, and corresponded to the intensity at which the value of the differentiated equation was equal to zero [75]. (iii) The sine model data analysis approach was used in 8 studies (7.1%). This data analysis approach is based on an specific equation that includes dilatation, symmetry, and translation as three independent variables representing the main modulations of the fat oxidation curve [75].

Time of the day when the test was performed

The time of the day when the graded exercise protocol was performed was different across the studies selected: (i) 84 studies (80.8%) were conducted in the morning, (ii) 5 studies were performed in the afternoon (4.8%), and (iii) 2 studies (1.9%) were conducted in the evening.

Acute nutritional status

The fasting time before the incremental graded exercise protocols varied across the studies selected (Table 3). Moreover, the meal established before the incremental graded exercise protocol were the following: (i) breakfast, lunch, and dinner before the incremental graded exercise protocol were standardized in 17 studies (15.2%), (ii) dinner before the incremental graded exercise protocol was standardized in 5 studies (4.5%), (iii) breakfast before the incremental graded exercise protocol was standardized in 5 studies (4.5%), (iv) non-standard meal before the incremental graded exercise protocol was established in 4 studies (3.6%), (v) breakfast and lunch before the incremental graded exercise protocol was standardized in 2 studies (1.8%), and (vi) lunch before the incremental graded exercise protocol was standardized in 1 study (0.9%).

Testing days for MFO/Fat_{max} and VO_2max assessment

The estimation of MFO/Fat_{max} by an incremental graded exercise protocol cannot be assessed if the VO_2max has not been previously determined. Therefore, the MFO/Fat_{max} and VO_2max can be assessed in the same day or in separate days. A total of 69 studies (61.6%) assessed MFO/Fat_{max} and VO_2max the same day, while a total of 43 studies (38.4%) assessed MFO/Fat_{max} and VO_2max in separate days.

Table 3. Fasting time conditions identified across the studies included in the systematic review

Fasting time	% of articles
Overnight fasting	26.7%
Fasting state of 12 hours	16.1%
Fasting state of 10 hours	10.7%
Fasting state of 4 hours	7.1%
Fasting state of 3 hours	6.3%
Fasting state of 10-12 hours	5.4%
Fasting state of 2 hours	5.4%
Fasting state of <1 hour	1.8%
Fasting state of 11 hours	1.8%
Fasting state of 8 hours	0.9%
Fasting state of 9-13 hours	0.9%
Fasting state of at least 5 hours	0.9%
Fasting state of at least 8 hours	0.9%
Fasting state of 3-12 hours	0.9%
Fasting state of 1 hour	0.9%
Fasting state of 8-12 hours	0.9%
Fasting state of 5 to 6 hours	0.9%
Fasting state of at least 6 hours	0.9%

DISCUSSION

The number of studies investigating MFO and Fat_{max} has grown enormously during the last years (see Figure 3). Besides individual's biological characteristics [20], there are several factors that potentially affect MFO and Fat_{max} estimation related to data collection and analysis approach (i.e. ergometer type, metabolic cart used, warm-up duration and intensity, stage duration and intensities imposed in the graded exercise protocol, time interval selected for data analysis, stoichiometric equation selected to estimate fat oxidation, data analysis approach, time of the day when the test was performed, and fasting time/previous meal before the test,

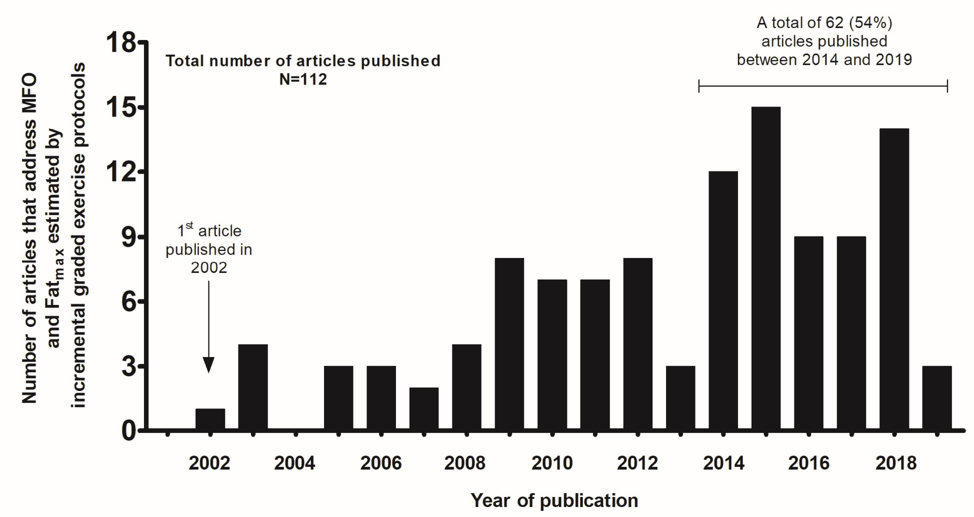

Figure 3. **N**umber of articles that address maximal fat oxidation (MFO) and the intensity that elicit MFO (Fatmax) estimated by incremental graded exercise protocols.

and testing days for MFO/ Fat_{max} and VO_2max assessment.).

We observed that a number of studies did not report information about key methodological issues related to the assessment of MFO and Fat_{max} (Figure 2). In addition, a high inter-study heterogeneity was observed in each key methodological issue. Thus, adequate across-study comparisons are not possible. Therefore, it is necessary to provide recommendations/guidelines about MFO and Fat_{max} data collection and processing to help harmonize future studies and make comparisons across studies possible.

Ergometer type

A number of previous studies have investigated whether the application of different ergometer types influences MFO and Fat_{max} in individuals with different biological characteristics [88,91,97,98,123,130–134]. Most of them compared a graded exercise protocol performed on a cyclo-ergometer vs. on a treadmill (walking/running). The findings obtained are not conclusive. While two studies reported greater MFO during walking/running on a treadmill than cycling on a cyclo-ergometer in healthy and moderately trained men with ~23 years of age (0.65±0.05 vs. 0.47±0.05 g/minute) [123] and in children aged from 8 to 13 (0.24±0.06 vs. 0.19±0.05 g/minute) [91], two other studies did not find significant differences in moderately trained individuals of both sexes aged ~29 (0.48±0.04 vs. 0.44±0.05 g/min) [98] and in healthy men and women aged 22 to 29 (0.50±0.19 vs. 0.47±0.22 g/minute) [88].

Furthermore, different results have been obtained when comparing treadmill vs. cyclo-ergometer graded exercise protocols in Fat_{max} across populations with different biological characteristics. Zakrzewski et al. [91] found higher Fat_{max} walking/running on a treadmill than cycling on a cyclo-ergometer in children (59±13 vs. 51±7% of VO_2max), which is in agreement with a study conducted in moderately trained men and women (57±2 vs. 44±3% of VO_2max) [98]. However, no differences in Fat_{max} comparing treadmill vs. cyclo-ergometer graded test protocols were reported in a similar individual population (59±3 vs. 62±3% of VO_2max) [123], and also in healthy men and women (49±20 vs. 50±15% of VO_2max) [88]. It has recently been suggested that these contradictory results can be related to the intraindividual variability and to the different data collection and analysis approaches used in the MFO and Fat_{max} determination [7,20]. Moreover, different ergometer models (e.g. electromagnetic vs mechanically braked cycle ergometer) have been used across studies not giving specific information about it in almost all cases, which could impact on MFO and Fat_{max} data. Thus, we encourage authors to describe the key methodological issues in detail, and also to compare sex-, age- and training-status-matched group of individuals [7].

Metabolic cart used

We registered a total of 14 different metabolic carts, yet there is no available information about the validity and reliability of these metabolic carts to measure MFO and FAT_{max}. The breath-by-breath systems are capable of measuring metabolic gas exchange during exercise but most of the commercially available metabolic carts have shown a wide inter-day reliability (~10%) that is clinically unacceptable [135]. Consequently, no gold standard has been well-recognised. However, assuming that differential monitor-specific deviations are one of the determinants for the lack of accuracy, comparability, and transferability of results obtained by IC, a post-calorimetric correction (i.e. correcting the measurements by artificial infusion of gases) was proposed. Interestingly, it was shown that this correction improved the comparability and reliability of the Vmax N29 metabolic cart and the Deltatrac Metabolic Monitor [136]. Galgani et al. [137] reported that the application of this post-calorimetric procedure appears to improve data quality in terms of substrate oxidation estimation during resting metabolic rate. Moreover, another key issue that should be carefully considered is that some metabolic cart systems assume constants atmospheric air factors, automatically correcting the registered VO_2 and VCO_2 concentrations. This fact could also modify the MFO and Fat_{max} assessment [138]. Therefore, the comparison between studies that used different metabolic carts to determine MFO and Fat_{max} should be considered with caution. Future studies are needed to investigate whether the application of a post-calorimetric procedure provides an accurate, valid, and reliable measurement of MFO and Fat_{max} in different metabolic carts. Furthermore, although a minor percentage of portable metabolic cart have been used in previous studies, it should be investigated whether the MFO and Fat_{max} estimations are similar using stationary vs. portable metabolic carts.

Warm-up protocol

Regarding the warm-up protocols, the duration ranges from 3 minutes to 10 minutes, with 3-5 minutes the most common duration. Warm-up intensity is commonly prescribed as a function of VO_2max (ranging from 10% to 60%) or as a predetermined external load (ranging from 6 to 100 watts in cyclo-ergometer protocols and from 2.5 to 7 km/h in treadmill protocols). It is important to considerer that if the selected warm-up intensity is too high, in certain populations a potential carry over effects could be produced, affecting MFO and Fat_{max} estimations. To our knowledge, there is no study comparing the effect of different warm-up durations or intensities on the MFO and Fat_{max} estimations. However, considering that Hajoglou et al. [139] reported that a specific warm-up protocol improved endurance performance more than no warm-up and other warm-up protocol in well-trained road cyclists, further studies are needed to clarify whether the selection of specific warm-up protocols for individuals with certain characteristic affects MFO and Fat_{max} estimations.

Graded exercise protocol

Fat oxidation during exercise has traditionally been studied over few stages, which included two to four progressive exercise intensities [140–142], being the resolution to establish MFO and Fat_{max} quite limited [28]. In order to improve this resolution, a graded exercise protocol with 3-minute stages on a cyclo-ergometer was proposed by Achten et al. [8]. Since then, numerous graded exercise protocol variations have been employed to determine MFO and Fat_{max}. The main variations involve alterations in exercise intensities (e.g. increments of 35 W vs. 20 W) [8,143] or stage durations (e.g. stage duration of 1 minute vs. 10 minutes) [80,97]. Nowadays, there is no consensus about which is the best stage duration to estimate MFO and Fat_{max}. The application of too large workload increments and short stage durations may lose accuracy on MFO and Fat_{max} estimation and do not ensure that the participant reaches steady-state gas exchange measure in each stage. In contrast, too small workload increments and long stage durations imply an excessively long test affecting the MFO and Fat_{max} estimation. Therefore, taking into account the biological characteristics of the study participant´s seems to be crucial on the graded exercise protocol design. Further studies are needed to investigate this specific issue.

Time interval for data selection and analysis

It seems clear that to reach a steady-state gaseous exchange (VO_2 and VCO_2) a key requirement is to accurately measure substrate metabolism through IC [144,145]. In this context, previous studies have suggested that individuals with low cardiorespiratory fitness need longer time periods to reach the steady-state gaseous exchange [146,147]. Thus, the application of a 3-minute stage duration or less could not be enough to attain the steady-state gaseous exchange in specific populations. A recent study showed that graded exercise protocols using a 4-minute stage duration are sufficient to establish steady-state gaseous exchange in individuals with low levels of cardiorespiratory fitness [28]. They also suggested that no differences were obtained comparing 4-minutes vs. 6-, 8-minutes stages, thus they recommended to use a 4-minutes stage duration, despite this stage duration could may systematically produce higher variability in gas exchange, and, consequently, in MFO and Fat_{max} [28]. However, although some studies have mentioned that it is necessary to reach a steady-state gaseous exchange during each graded exercise protocol stage to get a MFO and Fat_{max} valid measures [11,40,113,143,47,57,65,86–88,92,110], most of them did not report a detailed checking analysis. Therefore, we suggest using a graded exercise protocol with a 4-minute stage duration, using the last 60 seconds time interval for data analysis in low cardiorespiratory fitness individuals. Further studies are needed to know whether this recommendation is applicable for individuals with different cardiorespiratory fitness levels, and also to determine whether sex, age, and/or health status may play a role in this.

Stoichiometric equation selected to estimate fat oxidation

We found a total of 5 different stoichiometric equations to estimate fat oxidation across the studies selected from gas exchange data. A recent study suggested that further inter-study discrepancies in MFO and Fat_{max} can be attributed to the stoichiometric equation applied to calculate fuel oxidation [44]. This suggestion concurred with previous studies which proposed that the use of different stochiometric equations hinders inter-study comparisons [7,119]. To our knowledge, there are no studies that compare the estimation of fat oxidation through different stochiometric equations, and thus there is a need to investigate whether the use of different stochiometric equations provide similar or distinct MFO and Fat_{max} estimations. Moreover, despite the existence of studies estimating MFO through stochiometric equations, there are no investigations that evaluate the contribution of protein oxidation during the incremental graded exercise protocol.

Data analysis approach

We found a total of 3 different data analysis approaches to estimate MFO and Fat_{max} across the studies included in our systematic review. To date, only one study has investigated the reproducibility of MFO and Fat_{max} in recreationally trained males applying the measured-values data analysis approach, the polynomial curve data analysis approach, and the sine model data analysis approach [75]. They observed a high inter-individual variability (CV ~16%) in MFO and Fat_{max} regardless of the data analysis approach employed, despite the robust methodological design performed in their study [75]. However, the reproducibility of MFO and Fat_{max} in recreationally trained females and/or sedentary individuals applying the above-mentioned data analysis approaches has not been previously reported. Moreover, data analysis approaches that use fat oxidation data from stages between the lactate threshold and the critical power (e.g. polynomial curve data analysis approach or the sine model data analysis approach) should be considered cautiously, since it can take up to ~15-minutes to achieve steady-state gas exchange at these intensities. Therefore, further studies are needed to investigate which is the best data analysis approach depending of both the participants' biological characteristics, and also the graded exercise protocols methodological characteristics.

Time of the day when the test was performed

It is well known that numerous physiological processes are governed by a biological clock and have diurnal-variation patterns [148]. Indeed, previous studies showed that substrate oxidation in different conditions depends on the time of the day [149]. Nevertheless, little is known regarding the diurnal variations of substrate oxidation during exercise. To our knowledge, there is only two studies that investigated the diurnal variations of MFO and Fat_{max} concluding that both outcomes reached higher values in the evening than in the morning in untrained normal weight and obese men [94], and in trained male athletes [23]. Since we cannot extend these results to untrained and/or athletes' women, we recommend that the MFO and Fat_{max} assessment in the morning should not be compared with those measured in the afternoon and/or evening. We also encourage authors to precisely describe the time of the day at which the MFO and Fat_{max} is determined in order to avoid inappropriate and inaccurate comparisons.

Acute nutritional status

Our review shows a high inter-study variability of the acute nutritional status to estimate MFO and Fat_{max}, although a recent narrative review emphasized that the fasting time and the previous meal intake exert an important influence on MFO and Fat_{max} values [7]. Achten & Jeukendrup showed that

75 g of glucose ingested 45 minutes before exercise induced a reduction of MFO and Fat_{max} compared with placebo in trained males [121]. Moreover, a recent study showed that the dietary intake of carbohydrates and fat exert an independent negative and positive influence on MFO and Fat_{max}, respectively in healthy men and women. Although Astorino et al. [143] proposed that objectively standardising activity and diet may help increase reliability of estimating MFO and Fat_{max}, it is important to note that the majority of studies reported that the pre-trial diet standardisation was self-reported, which could impact on MFO and Fat_{max} determination. Therefore, we encourage authors to precisely control the acute nutritional status when the MFO and Fat_{max} are determined. Nevertheless, more studies are needed to confirm: (i) whether different fasting times before the incremental graded exercise protocol imply different MFO and Fat_{max} values, and (ii) whether non-previously studied dietary pattern maintained for long time periods (e.g. Mediterranean diet) induces alterations of MFO and Fat_{max}.

Testing days for MFO/Fat_{max} and VO_2max assessment

Fat_{max} is commonly expressed as the percentage of VO_2max, thus, the determination of the VO_2max is an essential requirement to assess MFO/Fat_{max}. This issue has entailed an important methodological research question with logistic and practical implications: to assess MFO/Fat_{max} and VO_2max in the same day, or in separate days. To resolve this matter, Guadalupe-Grau et al.[59] performed two different testing days in young healthy men performing: (i) A maximal incremental graded exercise protocol on a cycle ergometer, starting at 60 W for 5 min, followed by 35 W increment every 3 min until the RER reached 1.0. After that, increments of 35 W every minute was applied until exhaustion, obtaining MFO/Fat_{max} and VO_2max in the same day. (ii) A maximal graded exercise protocol on a cycloergometer starting with a 6-min warm-up at 60 W, followed by progressive increases of 35 W each minute until exhaustion, obtaining VO_2max. No significant differences were observed in VO_2max data measured by either day one or two testing days [59]. Therefore, we suggest to determine MFO/Fat_{max} and VO_2max in the same day for logistic and practical reasons in healthy young men. However, further studies are needed to know whether this recommendation is applicable for women, and/or other populations with different biological characteristics (i.e. trained vs. untrained, younger vs. older, etc.).

Limitations

Certain limitations need to be acknowledged. One limitation is that for certain population groups the number of studies was small, thus the recommendation and future directions outlined should be updated when more studies are published. Another point to note is that this review did not include studies conducted in patients.

CONCLUSIONS

Based on the findings of this systematic review, we suggest that researches measuring MFO and Fat_{max} using graded exercise protocols by IC should consider some key methodological issues that can considerably affect the accuracy, validity, and reliability of the measurement: (i) ergometer type, (ii) metabolic cart used, (iii) warm-up protocol (duration and intensity), (iv) graded exercise protocol (stage duration and intensities imposed), (v) time interval selected for data analysis, (vi) stoichiometric equation selected to estimate fat oxidation, (vii) data analysis approach, (viii) time of the day when the test was performed, (ix) acute nutritional status (fasting time and standard meal conditions established before the incremental graded exercise protocol) and (x) testing days for MFO/Fat_{max} and VO_2max assessment. Likewise, when comparing different studies, it is important to check whether the above-mentioned key methodological issues are similar in such studies to avoid ambiguous and unacceptable comparisons. Further studies are needed to develop detailed guidelines for MFO and Fat_{max} assessment using graded exercise protocols by IC.

Perspectives

Besides the individual's biological characteristics there are numerous factors that could affect MFO and Fat_{max} estimation related to data collection and analysis approach. Some key methodological issues should be considered by researches measuring MFO and Fat_{max} using graded exercise protocols by IC. These include: (i) ergometer type, (ii) metabolic cart used, (iii) warm-up protocol, (iv) graded exercise protocol, (v) time interval selected for data analysis, (vi) stoichiometric equation selected to estimate fat oxidation, (vii) data analysis approach, (viii) time of the day when the test was performed, (ix) acute nutritional status, and (x) testing days for MFO/Fat_{max} and VO_2max assessment.

The results of the current systematic review suggest that when comparing different studies, it is important to check whether the above-mentioned key methodological issues are similar in such studies to avoid ambiguous and unacceptable comparisons. Finally, an important aspect that should be considered when the MFO estimation of different studies are compared is whether MFO has been expressed in absolute values vs. relative values (g/min, $g/kg_{bodyweight}$/min, or $g/kg_{leanmass}$/min). This fact allows to compare data considering several key confounder variables such as sex, weight, and body composition.

REFERENCES

1 Elagizi A, Kachur S, Lavie CJ, Carbone S, Pandey A, Ortega F *et al.* An Overview and Update on Obesity and the Obesity Paradox in Cardiovascular Diseases. *Prog Cardiovasc Dis* 2018; **61**: 142–150.

2 Abarca-Gómez L, Abdeen ZA, Hamid ZA, Abu-Rmeileh NM, Acosta-Cazares B, Acuin C *et al.* Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: a pooled analysis of 2416 population-based measurement studies in 128·9 million children, adolescents, and adults. *Lancet* 2017; **390**: 2627–2642.

3 Galgani J, Ravussin E. Energy metabolism, fuel selection and body weight regulation. *Int J Obes (Lond)* 2008; **32 Suppl 7**: S109-19.

4 Seidell JC, Muller DC, Sorkin JD, Andres R. Fasting respiratory exchange ratio and resting metabolic rate as predictors of weight gain: the Baltimore Longitudinal Study on Aging. *Int J Obes Relat Metab Disord* 1992; **16**: 667–74.

5 Goodpaster BH, Sparks LM. Metabolic Flexibility in Health and Disease. *Cell Metab* 2017; **25**: 1027–1036.

6 Pérez-Martin A, Dumortier M, Raynaud E, Brun JF, Fédou C, Bringer J *et al.* Balance of substrate oxidation during submaximal exercise in lean and obese people. *Diabetes Metab* 2001; **27**: 466–74.

7 Maunder E, Plews DJ, Kilding AE. Contextualising Maximal Fat Oxidation During Exercise: Determinants and Normative Values. *Front Physiol* 2018; **9**: 1–13.

8 Achten J, Gleeson M, Jeukendrup AE. Determination of the exercise intensity that elicits maximal fat oxidation. *Med Sci Sports Exerc* 2002; **34**: 92–97.

9 Ara I, Larsen S, Stallknecht B, Guerra B, Morales-Alamo D, Andersen JL *et al.* Normal mitochondrial function and increased fat oxidation capacity in leg and arm muscles in obese humans. *Int J Obes (Lond)* 2011; **35**: 99–108.

10 Dandanell S, Husted K, Amdisen S, Vigelsø A, Dela F, Larsen S *et al.* Influence of maximal fat oxidation on long-term weight loss maintenance in humans. *J Appl Physiol* 2017; **123**: 267–274.

11 Dériaz O, Dumont M, Bergeron N, Després JP, Brochu M, Prud'homme D. Skeletal muscle low attenuation area and maximal fat oxidation rate during submaximal exercise in male obese individuals. *Int J Obes Relat Metab Disord* 2001; **25**: 1579–84.

12 Astorino TA. Is the ventilatory threshold coincident with maximal fat oxidation during submaximal exercise in women? *J Sports Med Phys Fitness* 2000; **40**: 209–216.

13 Larsen S, Ara I, Rabol R, Andersen JL, Boushel R, Dela F *et al.* Are substrate use during exercise and mitochondrial respiratory capacity decreased in arm and leg muscle in type 2 diabetes? *Diabetologia* 2009; **52**: 1400–1408.

14 Nordby P, Saltin B, Helge JW. Whole-body fat oxidation determined by graded exercise and indirect calorimetry: a role for muscle oxidative capacity? *Scand J Med Sci Sports* 2006; **16**: 209–14.

15 Lima-Silva AE, Bertuzzi RCM, Pires FO, Gagliardi JFL, Barros R V., Hammond J *et al.* Relationship between training status and maximal fat oxidation rate. *J Sport Sci Med* 2010; **9**: 31–35.

16 Schwindling S, Scharhag-Rosenberger F, Kindermann W, Meyer T. Limited benefit of Fatmax-test to derive training prescriptions. *Int J Sports Med* 2014; **35**: 280–285.

17 Chenevière X, Borrani F, Sangsue D, Gojanovic B, Malatesta D. Gender differences in whole-body fat oxidation kinetics during exercise. *Appl Physiol Nutr Metab* 2011; **36**: 88–95.

18 Fletcher G, Eves FF, Glover EI, Robinson SL, Vernooij CA, Thompson JL *et al.* Dietary intake is independently associated with the maximal capacity for fat oxidation during exercise. *Am J Clin Nutr* 2017; **105**: 864–872.

19 Venables MC, Achten J, Jeukendrup AE. Determinants of fat oxidation during exercise in healthy men and women: a cross-sectional study. *J Appl Physiol* 2005; **98**: 160–167.

20 Amaro-Gahete FJ, Ruiz JR. Methodological issues related to maximal fat oxidation rate during exercise. *Eur J Appl Physiol* 2018; **118**: 2029–2031.

21 Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JPA *et al.* The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. *J Clin Epidemiol* 2009; **62**: e1-34.

22 Methley AM, Campbell S, Chew-Graham C, McNally R, Cheraghi-Sohi S. PICO, PICOS and SPIDER: A comparison study of specificity and sensitivity in three search tools for qualitative systematic reviews. *BMC Health Serv Res* 2014; **14**.

23 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; : 1–20.

24 Özgünen KT, Özdemir Ç, Korkmaz-Eryılmaz S, Kılcı A, Günaştı Ö, Kurdak SS. A Comparison of the Maximal Fat Oxidation Rates of Three Different Time Periods in The Fatmax Stage. *J Sports Sci Med* 2019; **18**: 44–51.

25 Soria M, Ansón M, Lou-Bonafonte JM, Andrés-Otero MJ, Puente JJ, Escanero J. Fat Oxidation Rate as a Function of Plasma Lipid and Hormone Response in Endurance Athletes. *J strength Cond Res* 2019; **00**: 1–10.

26 Astorino TA, Schubert MM. Changes in fat oxidation in response to various regimes of high intensity interval training (HIIT). *Eur J Appl Physiol* 2018; **118**: 51–63.

27 Cancino Ramirez J, Soto Sanchez J, Zbinden Foncea H, Moreno Gonzalez M, Leyton Dinamarca B, Gonzalez Rojas L. Cardiorespiratory fitness and fat oxidation during exercise as protective factors for insulin resistance in sedentary women with overweight or obesity. *Nutr Hosp* 2018; **0**: 312–317.

28 Chrzanowski-Smith OJ, Edinburgh RM, Betts JA, Stokes KA, Gonzalez JT. Evaluation of a graded exercise test to determine peak fat oxidation in individuals with low cardiorespiratory fitness. *Appl Physiol Nutr Metab* 2018.

29 Cipryan L, Plews DJ, Ferretti A, Maffetone PB, Laursen PB. Effects of a 4-Week Very Low-Carbohydrate Diet on High-Intensity Interval Training Responses. *J Sports Sci Med* 2018; **17**: 259–268.

30 Dandanell S, Meinild-Lundby A-K,

Andersen AB, Lang PF, Oberholzer L, Keiser S *et al.* Determinants of maximal whole-body fat oxidation in elite cross-country skiers: Role of skeletal muscle mitochondria. *Scand J Med Sci Sports* 2018; **28**: 2494–2504.

31 Desai T, Bottoms L, Roberts M. The effects of Montmorency tart cherry juice supplementation and FATMAX exercise on fat oxidation rates and cardio-metabolic markers in healthy humans. *Eur J Appl Physiol* 2018; **118**: 2523–2539.

32 Guadalupe-Grau A, Fernandez-Elias VE, Ortega JF, Dela F, Helge JW, Mora-Rodriguez R. Effects of 6-month aerobic interval training on skeletal muscle metabolism in middle-aged metabolic syndrome patients. *Scand J Med Sci Sports* 2018; **28**: 585–595.

33 Gutierrez-Hellin J, Del Coso J. Dose-Response Effects of p-Synephrine on Fat Oxidation Rate During Exercise of Increasing Intensity. *Phytother Res* 2018; **32**: 370–374.

34 Gutierrez-Hellin J, Del Coso J. Effects of p-Synephrine and Caffeine Ingestion on Substrate Oxidation during Exercise. *Med Sci Sports Exerc* 2018.

35 Larsen S, Vigelsø A, Dandanell S, Prats C, Dela F, Helge JW. Simvastatin-Induced Insulin Resistance May Be Linked to Decreased Lipid Uptake and Lipid Synthesis in Human Skeletal Muscle: the LIFESTAT Study. *J Diabetes Res* 2018; **2018**: 9257874.

36 Stein E, Silva IM, Dorneles GP, Lira FS, Romão P, Peres A. Reduced Fat Oxidation During Exercise in Post-Menopausal Overweight-Obese Women with Higher Lipid Accumulation Product Index. *Exp Clin Endocrinol Diabetes* 2018.

37 Tan S, Du P, Zhao W, Pang J, Wang J. Exercise Training at Maximal Fat Oxidation Intensity for Older Women with Type 2 Diabetes. *Int J Sports Med* 2018; **39**: 374–381.

38 Vest S, Frandsen J, Larsen S, Dela F, Helge J. Peak Fat Oxidation is not Independently Related to Ironman Performance in Women. *Int J Sports Med* 2018; **39**: 916–923.

39 Yokoyama H, Takeda R, Kawai E, Ota A, Morita E, Imai D *et al.* Inhibitory Effects of Intranasal Administration of Insulin on Fat Oxidation during Exercise Are Diminished in Young Overweight Individuals. *J Clin Med* 2018; **7**: 308.

40 Dandanell S, Præst CB, Søndergård SD, Skovborg C, Dela F, Larsen S *et al.* Determination of the exercise intensity that elicits maximal fat oxidation in individuals with obesity. *Appl Physiol Nutr Metab* 2017; **42**: 405–412.

41 Durkalec-Michalski K, Zawieja EE, Zawieja BE, Podgorski T, Jurkowska D, Jeszka J. Influence of low versus moderate glycemic index of diet on substrate oxidation and energy expenditure during incremental exercise in endurance athletes: a randomized counterbalanced cross-over trial. *Int J Food Sci Nutr* 2017; : 1–12.

42 Frandsen J, Vest SD, Larsen S, Dela F, Helge JW. Maximal Fat Oxidation is Related to Performance in an Ironman Triathlon. *Int J Sports Med* 2017; **38**: 975–982.

43 Morville T, Rosenkilde M, Munch-Andersen T, Andersen PR, Kjær Groenbæk K, Helbo S *et al.* Repeated Prolonged Exercise Decreases Maximal

Fat Oxidation in Older Men. *Med Sci Sports Exerc* 2017; **49**: 308–316.

44 Peric R, Meucci M, Bourdon PC, Nikolovski Z. Does the aerobic threshold correlate with the maximal fat oxidation rate in short stage treadmill tests? *J Sports Med Phys Fitness* 2017.

45 Ponce González JG, Guadalupe-Grau A, Rodríguez-González FG, Torres-Peralta R, Morales-Alamo D, Rodríguez-García L *et al.* Androgen receptor gene polymorphisms and maximal fat oxidation in healthy men. A longitudinal study. *Nutr Hosp* 2017; **34**: 1089–1098.

46 Randell RK, Rollo I, Roberts TJ, Dalrymple KJ, Jeukendrup AE, Carter JM. Maximal Fat Oxidation Rates in an Athletic Population. *Med Sci Sports Exerc* 2017; **49**: 133–140.

47 Andersson-Hall U, Pettersson S, Edin F, Pedersen A, Malmodin D, Madsen K. Metabolism and whole-body fat oxidation following postexercise carbohydrate or protein intake. *Int J Sport Nutr Exerc Metab* 2016; **28**: 37–45.

48 Croci I, Byrne NM, Chachay VS, Hills AP, Clouston AD, O'Moore-Sullivan TM *et al.* Independent effects of diet and exercise training on fat oxidation in non-alcoholic fatty liver disease. *World J Hepatol* 2016; **8**: 1137–1148.

49 De Souza Silveira R, Carlsohn A, Langen G, Mayer F, Scharhag-Rosenberger F. Reliability and day-to-day variability of peak fat oxidation during treadmill ergometry. *J Int Soc Sports Nutr* 2016; **13**: 1–7.

50 Gutiérrez-Hellín J, Del Coso J. Acute p-synephrine ingestion increases fat oxidation rate during exercise. *Br J Clin Pharmacol* 2016; : 362–368.

51 Kim H-K, Ando K, Tabata H, Konishi M, Takahashi M, Nishimaki M *et al.* Effects of Different Intensities of Endurance Exercise in Morning and Evening on the Lipid Metabolism Response. *J Sports Sci Med* 2015; **15**: 467–476.

52 Peric R, Meucci M, Nikolovski Z. Fat Utilization During High-Intensity Exercise: When Does It End? *Sport Med - open* 2016; **2**: 35.

53 Robinson S, Chambers E, Fletcher G, Wallis G. Lipolytic Markers, Insulin and Resting Fat Oxidation are Associated with Maximal Fat Oxidation. *Int J Sports Med* 2016; **37**: 607–613.

54 Tan S, Wang J, Cao L. Exercise training at the intensity of maximal fat oxidation in obese boys. *Appl Physiol Nutr Metab* 2016; **41**: 49–54.

55 Tan S, Wang J, Cao L, Guo Z, Wang Y. Positive effect of exercise training at maximal fat oxidation intensity on body composition and lipid metabolism in overweight middle-aged women. *Clin Physiol Funct Imaging* 2016; **36**: 225–30.

56 Alkhatib A, Seijo M, Larumbe E, Naclerio F. Acute effectiveness of a 'fat-loss' product on substrate utilization, perception of hunger, mood state and rate of perceived exertion at rest and during exercise. *J Int Soc Sports Nutr* 2015; **12**: 44.

57 Besnier F, Lenclume V, Gérardin P, Fianu A, Martinez J, Naty N *et al.* Individualized exercise training at maximal fat oxidation combined with fruit and vegetable-rich diet in overweight or obese women: The LIPOXmax-réunion randomized controlled trial. *PLoS One* 2015; **10**: 27–40.

58 Borel B, Coquart J, Boitel G, Duhamel A,

Matran R, Delsart P *et al.* Effects of Endurance Training at the Crossover Point in Women with Metabolic Syndrome. *Med Sci Sports Exerc* 2015; **47**: 2380–2388.

59 Guadalupe-Grau A, Plenge U, Helbo S, Kristensen M, Andersen PR, Fago A *et al.* Effects of an 8-weeks erythropoietin treatment on mitochondrial and whole body fat oxidation capacity during exercise in healthy males. *J Sports Sci* 2015; **33**: 570–578.

60 Isacco L, Thivel D, Pereira B, Duclos M, Boisseau N. Maximal fat oxidation, but not aerobic capacity, is affected by oral contraceptive use in young healthy women. *Eur J Appl Physiol* 2015; **115**: 937–45.

61 Lanzi S, Codecasa F, Cornacchia M, Maestrini S, Capodaglio P, Brunani A *et al.* Short-term HIIT and Fat max training increase aerobic and metabolic fitness in men with class II and III obesity. *Obesity (Silver Spring)* 2015; **23**: 1987–1994.

62 Marzouki H, Gmada N, Farhani Z, Hssin N, Shephard R, Bouhlel E. Crossover and maximal fat oxidation points during running and cycling in sedentary subjects. *Sci Sports* 2015; **30**: 196–203.

63 Mendelson M, Michallet A-S, Monneret D, Perrin C, Esteve F, Lombard PR *et al.* Impact of exercise training without caloric restriction on inflammation, insulin resistance and visceral fat mass in obese adolescents. *Pediatr Obes* 2015; **10**: 311–319.

64 Mohebbi H, Nourshahi M, Ghasemikaram M, Safarimosavi S. Effects of exercise at individual anaerobic threshold and maximal fat oxidation intensities on plasma levels of nesfatin-1 and metabolic health biomarkers. *J Physiol Biochem* 2015; **71**: 79–88.

65 Nordby P, Rosenkilde M, Ploug T, Westh K, Feigh M, Nielsen NB *et al.* Independent effects of endurance training and weight loss on peak fat oxidation in moderately overweight men: a randomized controlled trial. *J Appl Physiol* 2015; **118**: 803–10.

66 Robinson SL, Hattersley J, Frost GS, Chambers ES, Wallis GA. Maximal fat oxidation during exercise is positively associated with 24-hour fat oxidation and insulin sensitivity in young, healthy men. *J Appl Physiol* 2015; **118**: 1415–1422.

67 Rosenkilde M, Reichkendler MH, Auerbach P, Bonne TC, Sjödin A, Ploug T *et al.* Changes in peak fat oxidation in response to different doses of endurance training. *Scand J Med Sci Sport* 2015; **25**: 41–52.

68 Suk MH, Moon Y-J, Park SW, Park C-Y, Shin YA. Maximal Fat Oxidation Rate during Exercise in Korean Women with Type 2 Diabetes Mellitus. *Diabetes Metab J* 2015; **39**: 328–34.

69 Wang J, Tan S, Cao L. Exercise training at the maximal fat oxidation intensity improved health-related physical fitness in overweight middle-aged women. *J Exerc Sci Fit* 2015; **13**: 111–116.

70 Alkhatib A. Yerba Maté (Illex Paraguariensis) ingestion augments fat oxidation and energy expenditure during exercise at various submaximal intensities. *Nutr Metab (Lond)* 2014; **11**: 42.

71 Alvehus M, Boman N, Soderlund K, Svensson MB, Buren J. Metabolic adaptations in skeletal muscle, adipose tissue, and whole-body oxidative capacity in response to resistance training. *Eur J*

Appl Physiol 2014; **114**: 1463–1471.

72 Astorino TA, Schubert MM. Individual responses to completion of short-term and chronic interval training: a retrospective study. *PLoS One* 2014; **9**: e97638.

73 Blaize AN, Potteiger JA, Claytor RP, Noe DA. Body fat has no effect on the maximal fat oxidation rate in young, normal, and overweight women. *J strength Cond Res* 2014; **28**: 2121–6.

74 Croci I, Hickman IJ, Wood RE, Borrani F, Macdonald G a, Byrne NM. Fat oxidation over a range of exercise intensities: fitness versus fatness. *Appl Physiol Nutr Metab* 2014; **39**: 1352–1359.

75 Croci I, Borrani F, Byrne NM, Byrne N, Wood RE, Wood R *et al.* Reproducibility of Fatmax and fat oxidation rates during exercise in recreationally trained males. *PLoS One* 2014; **9**: e97930.

76 Chu L, Riddell MC, Schneiderman JE, McCrindle BW, Hamilton JK. The effect of puberty on fat oxidation rates during exercise in overweight and normal-weight girls. *J Appl Physiol* 2014; **116**: 76–82.

77 Isacco L, Thivel D, Duclos M, Aucouturier J, Boisseau N. Effects of adipose tissue distribution on maximum lipid oxidation rate during exercise in normal-weight women. *Diabetes Metab* 2014; **40**: 215–219.

78 Jabbour G, O'Loughlin J, Sabiston C, Tremblay A, Mathieu M-E. Increased lipid oxidation during exercise in obese pubertal girls: a QUALITY study. *Obesity (Silver Spring)* 2014; **22**: E85-90.

79 Lanzi S, Codecasa F, Cornacchia M, Maestrini S, Salvadori A, Brunani A *et al.* Fat oxidation, hormonal and plasma metabolite kinetics during a submaximal incremental test in lean and obese adults. *PLoS One* 2014; **9**: e88707.

80 Takagi S, Sakamoto S, Midorikawa T, Konishi M, Katsumura T. Determination of the exercise intensity that elicits maximal fat oxidation in short-time testing. *J Sports Sci* 2014; **32**: 175–182.

81 Astorino TA, Schubert MM, Palumbo E, Stirling D, McMillan DW. Effect of two doses of interval training on maximal fat oxidation in sedentary women. *Med Sci Sports Exerc* 2013; **45**: 1878–86.

82 Croci I, Byrne NM, Choquette S, Hills AP, Chachay VS, Clouston AD *et al.* Whole-body substrate metabolism is associated with disease severity in patients with non-alcoholic fatty liver disease. *Gut* 2013; **62**: 1625–1633.

83 Décombaz J, Grathwohl D, Pollien P, Schmitt JAJ, Borrani F, Lecoultre V. Effect of short-duration lipid supplementation on fat oxidation during exercise and cycling performance. *Appl Physiol Nutr Metab* 2013; **38**: 766–72.

84 Chenevière X, Borrani F, Droz D, Gojanovic B, Malatesta D. Effects of 2 different prior endurance exercises on whole-body fat oxidation kinetics: light vs. heavy exercise. *Appl Physiol Nutr Metab* 2012; **37**: 955–964.

85 Desplan M, Brun J-F, Pillard F, Fedou C, Prefaut C, Mercier J *et al.* Decreased fat oxidation during exercise in severe obstructive sleep apnoea syndrome. *Diabetes Metab* 2012; **38**: 236–242.

86 Gmada N, Marzouki H, Haboubi M, Tabka Z, Shephard RJ, Bouhlel E. Crossover and maximal fat-oxidation points in sedentary healthy subjects: methodological issues. *Diabetes Metab* 2012; **38**: 40–5.

87 Makni E, Moalla W, Trabelsi Y, Lac G, Brun JF, Tabka Z *et al.* Six-minute walking test predicts maximal fat oxidation in obese children. *Int J Obes (Lond)* 2012; **36**: 908–13.

88 Mendelson M, Jinwala K, Wuyam B, Levy P, Flore P. Can crossover and maximal fat oxidation rate points be used equally for ergocycling and walking/running on a track? *Diabetes Metab* 2012; **38**: 264–270.

89 Tsujimoto T, Sasai H, Miyashita M, Eto M, So R, Ohkubo H *et al.* Effect of weight loss on maximal fat oxidation rate in obese men. *Obes Res Clin Pract* 2012; **6**: e91–e174.

90 Zakrzewski JK, Tolfrey K. Acute effect of Fatmax exercise on the metabolism in overweight and nonoverweight girls. *Med Sci Sports Exerc* 2012; **44**: 1698–1705.

91 Zakrzewski JK, Tolfrey K. Comparison of fat oxidation over a range of intensities during treadmill and cycling exercise in children. *Eur J Appl Physiol* 2012; **112**: 163–171.

92 Chu L, Riddell MC, Takken T, Timmons BW. Carbohydrate intake reduces fat oxidation during exercise in obese boys. *Eur J Appl Physiol* 2011; **111**: 3135–41.

93 González-Haro C. Maximal fat oxidation rate and cross-over point with respect to lactate thresholds do not have good agreement. *Int J Sports Med* 2011; **32**: 379–85.

94 Mohebbi H, Azizi M. Maximal fat oxidation at the different exercise intensity in obese and normal weight men in the morning and evening. *J Hum Sport Exerc* 2011; **6**: 49–58.

95 Rynders CA, Angadi SS, Weltman NY, Gaesser GA, Weltman A. Oxygen uptake and ratings of perceived exertion at the lactate threshold and maximal fat oxidation rate in untrained adults. *Eur J Appl Physiol* 2011; **111**: 2063–8.

96 Zakrzewski J, Tolfrey K. Exercise protocols to estimate Fatmax and maximal fat oxidation in children. *Pediatr Exerc Sci* 2011; **23**: 122–35.

97 Capostagno B, Bosch A. Higher fat oxidation in running than cycling at the same exercise intensities. *Int J Sport Nutr Exerc Metab* 2010; **20**: 44–55.

98 Chenevière X, Malatesta D, Gojanovic B, Borrani F. Differences in whole-body fat oxidation kinetics between cycling and running. *Eur J Appl Physiol* 2010; **109**: 1037–45.

99 Del Coso J, Hamouti N, Ortega JF, Mora-Rodriguez R. Aerobic fitness determines whole-body fat oxidation rate during exercise in the heat. *Appl Physiol Nutr Metab = Physiol Appl Nutr Metab* 2010; **35**: 741–748.

100 Haufe S, Engeli S, Budziarek P, Utz W, Schulz-Menger J, Hermsdorf M *et al.* Determinants of exercise-induced fat oxidation in obese women and men. *Horm Metab Res = Horm und Stoffwechselforsch = Horm Metab* 2010; **42**: 215–221.

101 Rosenkilde M, Nordby P, Nielsen LB, Stallknecht BM, Helge JW. Fat oxidation at rest predicts peak fat oxidation during exercise and metabolic phenotype in overweight men. *Int J Obes (Lond)* 2010; **34**: 871–7.

102 Scharhag-Rosenberger F, Meyer T, Walitzek S, Kindermann W. Effects of one year aerobic endurance training on resting metabolic rate and exercise fat oxidation in previously untrained men and women. Metabolic endurance training

adaptations. *Int J Sports Med* 2010; **31**: 498–504.

103 Tolfrey K, Jeukendrup AE, Batterham AM. Group- and individual-level coincidence of the 'Fatmax' and lactate accumulation in adolescents. *Eur J Appl Physiol* 2010; **109**: 1145–1153.

104 Aucouturier J, Rance M, Meyer M, Isacco L, Thivel D, Fellmann N *et al.* Determination of the maximal fat oxidation point in obese children and adolescents: Validity of methods to assess maximal aerobic power. *Eur J Appl Physiol* 2009; **105**: 325–331.

105 Cheneviere X, Borrani F, Ebenegger V, Gojanovic B, Malatesta D. Effect of a 1-hour single bout of moderate-intensity exercise on fat oxidation kinetics. *Metabolism* 2009; **58**: 1778–1786.

106 Cheneviere X, Malatesta D, Peters EM, Borrani F. A mathematical model to describe fat oxidation kinetics during graded exercise. *Med Sci Sports Exerc* 2009; **41**: 1615–1625.

107 Meyer T, Folz C, Rosenberger F, Kindermann W. The reliability of fatmax. *Scand J Med Sci Sports* 2009; **19**: 213–21.

108 Mogensen M, Vind BF, Højlund K, Beck-Nielsen H, Sahlin K. Maximal lipid oxidation in patients with type 2 diabetes is normal and shows an adequate increase in response to aerobic training. *Diabetes, Obes Metab* 2009; **11**: 874–883.

109 Zunquin G, Theunynck D, Sesboue B, Arhan P, Bougle D. Evolution of fat oxidation during exercise in obese pubertal boys: clinical implications. *J Sports Sci* 2009; **27**: 315–318.

110 Zunquin G, Theunynck D, Sesboüé B, Arhan P, Bouglé D. Comparison of fat oxidation during exercise in lean and obese pubertal boys: clinical implications. *Br J Sports Med* 2009; **43**: 869–70.

111 Bogdanis GC, Vangelakoudi A, Maridaki M. Peak fat oxidation rate during walking in sedentary overweight men and women. *J Sport Sci Med* 2008; **1**: 525–531.

112 Michallet A-S, Tonini J, Regnier J, Guinot M, Favre-Juvin A, Bricout V *et al.* Methodological aspects of crossover and maximum fat-oxidation rate point determination. *Diabetes Metab* 2008; **34**: 514–523.

113 Riddell MC, Jamnik VK, Iscoe KE, Timmons BW, Gledhill N. Fat oxidation rate and the exercise intensity that elicits maximal fat oxidation decreases with pubertal status in young male subjects. *J Appl Physiol* 2008; **105**: 742–748.

114 Venables MC, Jeukendrup AE. Endurance training and obesity: effect on substrate metabolism and insulin sensitivity. *Med Sci Sports Exerc* 2008; **40**: 495–502.

115 Gonzalez-Haro C, Galilea PA, Gonzalez-de-Suso JM, Drobnic F, Escanero JF. Maximal lipidic power in high competitive level triathletes and cyclists. *Br J Sports Med* 2007; **41**: 23–28.

116 Kang J, Hoffman JR, Ratamess NA, Faigenbaum AD, Falvo M, Wendell M. Effect of exercise intensity on fat utilization in males and females. *Res Sports Med* 2007; **15**: 175–188.

117 Bennard P, Doucet E. Acute effects of exercise timing and breakfast meal glycemic index on exercise-induced fat oxidation. *Appl Physiol Nutr Metab = Physiol Appl Nutr Metab* 2006; **31**: 502–511.

118 Stisen AB, Stougaard O, Langfort J, Helge JW, Sahlin K, Madsen K. Maximal fat

oxidation rates in endurance trained and untrained women. *Eur J Appl Physiol* 2006; **98**: 497–506.

119 Bircher S, Knechtle B, Knecht H. Is the intensity of the highest fat oxidation at the lactate concentration of 2 mmol L(-1)? A comparison of two different exercise protocols. *Eur J Clin Invest* 2005; **35**: 491–498.

120 Brandou F, Savy-Pacaux AM, Marie J, Bauloz M, Maret-Fleuret I, Borrocoso S *et al.* Impact of high- and low-intensity targeted exercise training on the type of substrate utilization in obese boys submitted to a hypocaloric diet. *Diabetes Metab* 2005; **31**: 327–335.

121 Achten J, Jeukendrup AE. The effect of pre-exercise carbohydrate feedings on the intensity that elicits maximal fat oxidation. *J Sports Sci* 2003; **21**: 1017–24.

122 Achten J, Jeukendrup AE. Maximal fat oxidation during exercise in trained men. *Int J Sports Med* 2003; **24**: 603–8.

123 Achten J, Venables MC, Jeukendrup AE. Fat oxidation rates are higher during running compared with cycling over a wide range of intensities. *Metabolism* 2003; **52**: 747–752.

124 Brandou F, Dumortier M, Garandeau P, Mercier J, Brun JF. Effects of a two-month rehabilitation program on substrate utilization during exercise in obese adolescents. *Diabetes Metab* 2003; **29**: 20–27.

125 Frayn KN. Calculation of substrate oxidation rates in vivo from gaseous exchange. *J Appl Physiol* 1983; **55**: 628–34.

126 Péronnet F, Massicotte D. Table of nonprotein respiratory quotient: an update. *Can J Sport Sci* 1991; **16**: 23–9.

127 Jeukendrup AE, Wallis GA. Measurement of substrate oxidation during exercise by means of gas exchange measurements. *Int J Sports Med* 2005; **26 Suppl 1**: S28-37.

128 Brouwer E. On simple formulae for calculating the heat expenditure and the quantities of carbohydrate and fat oxidized in metabolism of men and animals, from gaseous exchange (Oxygen intake and carbonic acid output) and urine-N. *Acta Physiol Pharmacol Neerl* 1957; **6**: 795–802.

129 Elia M, Livesey G. Energy expenditure and fuel selection in biological systems: the theory and practice of calculations based on indirect calorimetry and tracer methods. *World Rev Nutr Diet* 1992; **70**: 68–131.

130 Arkinstall MJ, Bruce CR, Nikolopoulos V, Garnham AP, Hawley JA. Effect of carbohydrate ingestion on metabolism during running and cycling. *J Appl Physiol* 2001; **91**: 2125–2134.

131 Glass SC, Santos VJ, Armstrong D. The Effect of Mode of Exercise on Fat Oxidation during Exercise. *J Strength Cond Res* 1999; **13**: 29–34.

132 Lafortuna CL, Lazzer S, Agosti F, Busti C, Galli R, Mazzilli G *et al.* Metabolic responses to submaximal treadmill walking and cycle ergometer pedalling in obese adolescents. *Scand J Med Sci Sports* 2010; **20**: 630–637.

133 Snyder A, O'Hagan K, Clifford P, Hoffman M, Foster C. Exercise Responses to In-Line Skating: Comparisons to Running and Cycling. *Int J Sports Med* 1993; **14**: 38–42.

134 Thomas TR, Feiock CW, Araujo J. Metabolic responses associated with four modes of prolonged exercise. *J Sports Med*

Phys Fitness 1989; **29**: 77–82.

135 Schoeller DA. Making Indirect Calorimetry a Gold Standard for Predicting Energy Requirements for Institutionalized Patients. *J Am Diet Assoc* 2007; **107**: 390–392.

136 Schadewaldt P, Nowotny B, Straßburger K, Kotzka J, Roden M. Indirect calorimetry in humans: A postcalorimetric evaluation procedure for correction of metabolic monitor variability. *Am J Clin Nutr* 2013; **97**: 763–773.

137 Galgani JE, Castro-Sepulveda MA. Influence of a Gas Exchange Correction Procedure on Resting Metabolic Rate and Respiratory Quotient in Humans. *Obesity* 2017; **25**: 1941–1947.

138 Betts JA, Thompson D. Thinking outside the bag (not necessarily outside the lab). *Med Sci Sports Exerc* 2012; **44**: 2040; author reply 2041.

139 Hajoglou A, Foster C, De Koning JJ, Lucia A, Kernozek TW, Porcari JP. Effect of warm-up on cycle time trial performance. *Med Sci Sports Exerc* 2005; **37**: 1608–1614.

140 Bergman BC, Brooks GA. Respiratory gas-exchange ratios during graded exercise in fed and fasted trained and untrained men. *J Appl Physiol* 1999; **86**: 479–487.

141 Sidossis LS, Wolfe RR, Coggan AR. Regulation of fatty acid oxidation in untrained vs. trained men during exercise. *Am J Physiol* 1998; **274**: E510-5.

142 Romijn J a, Gastaldelli a, Horowitz JF, Endert E, Wolfe RR, Coyle E *et al.* Regulation in relation of endogenous fat and carbohydrate to exercise intensity and duration metabolism. *Endocrinol Metab* 1993; **28**: 380–391.

143 Astorino TA, Edmunds RM, Clark A, Gallant R, King L, Ordille GM *et al.* Change in maximal fat oxidation in response to different regimes of periodized high-intensity interval training (HIIT). *Eur J Appl Physiol* 2018; **117**: 745–755.

144 Shephard RJ, Aoyagi Y. Measurement of human energy expenditure, with particular reference to field studies: An historical perspective. *Eur J Appl Physiol* 2012; **112**: 2785–2815.

145 Macfarlane DJ. Open-circuit respirometry: a historical review of portable gas analysis systems. *Eur J Appl Physiol* 2017; **117**: 2369–2386.

146 Brun JF, Romain AJ, Mercier J. Lipoxmax ou oxydation maximale des lipides lors de l'exercice: Des mesures physiologiques aux applications cliniques. Réalités et incertitudes. *Sci Sport* 2011; **26**: 57–71.

147 Bordenave S, Flavier S, Fedou C, Brun JF, Mercier J. Exercise calorimetry in sedentary patients: procedures based on short 3 min steps underestimate carbohydrate oxidation and overestimate lipid oxidation. *Diabetes Metab* 2007; **33**: 379–384.

148 Gonnissen HKJ, Hulshof T, Westerterp-Plantenga MS. Chronobiology, endocrinology, and energy- and food-reward homeostasis. *Obes Rev* 2013; **14**: 405–416.

149 Froy O. Metabolism and circadian rhythms - Implications for obesity. *Endocr Rev* 2010; **31**: 1–24.

Impact of data analysis methods for maximal fat oxidation estimation during exercise in sedentary adults ***(Study 4)***

ABSTRACT

MFO and Fat_{max} are considered excellent markers of fat metabolism during exercise. Besides individual's biological characteristics (e.g. fed state, physical fitness level, sex, or age), data selection and analysis can affect MFO and Fatmax estimations, yet the effect is unknown. We investigated (i) the impact of using a pre-defined time interval on MFO and Fat_{max} estimation, and (ii) the impact of applying 2 different data analysis approaches (measured-values vs. polynomial-curve) on MFO and Fat_{max} estimations in sedentary adults. A total of 151 (97 women) sedentary adults aged 29.2 ± 13.2 years old participated in the study. We assessed MFO and Fatmax through a walking graded exercise test using indirect calorimetry. We pre-defined 13 different time intervals for data analysis, and the estimation of MFO and Fat_{max} were performed through the measured-values and the polynomial-curve data analysis approaches. There were significant differences in MFO across pre-defined time intervals methods ($P<0.001$) applying measured-values data analysis approach, while no statistical differences were observed when using polynomial-curve data analysis approach ($P=0.077$). There were no differences in Fat_{max} across pre-defined time intervals independently of the data analysis approach ($P\geq0.7$). We observed significant differences in MFO between measured-values and the polynomial-curve data analysis approaches across the time intervals methods selected (all $P\leq0.05$), and no differences were observed in Fat_{max} (all $P\geq0.2$). In conclusion, our results revealed that there are no differences in MFO and Fat_{max} across different time intervals methods selected using the polynomial-curve data analysis approach. We observed significant differences in MFO between measured-values vs. polynomial-curve data analysis approaches in all the study time intervals, whereas no differences were detected in Fatmax. Therefore, the use of polynomial-curve data analysis approach allows to compare MFO and Fat_{max} using different time intervals in sedentary adults.

BACKGROUND

A low capacity to oxidize fatty acids by whole body and skeletal muscle in the fasted state [1] or during exercise [2] is one of the most important factors related to metabolic diseases [3]. MFO and Fat_{max} are currently considered excellent markers of fat metabolism during exercise, and have been used to investigate alterations of substrate metabolism in obesity [4–7] and type II diabetes [8]. Moreover, MFO and Fat_{max} have been considered key factors in endurance sport performance [9].

During exercise fat oxidation increases as exercise intensity increases until fat oxidation peak (i.e. MFO) is reached at a certain intensity (i.e. Fat_{max}), and then fat oxidation begins to decline. From this exercise intensity onwards, carbohydrate oxidation becomes the primary energy source [10,11]. Interestingly, there is a high interindividual variability in fuel oxidation substrates during exercise, which depends on sex, age, or training status [4,10–13]. Besides these individual's biological characteristics, there are numerous factors known to affect both MFO and Fat_{max} estimation, including the graded exercise protocol applied [14,15], the exercise type [16], the metabolic cart used to register the VO_2 and VCO_2 release during the exercise protocol [13,17,18], and the stoichiometric equations selected to calculate fuel oxidation [14,17–20].

Previous studies have shown that data selection and analysis methods, when applied to IC data, exert an important influence on BMR and meal-induced thermogenesis estimations [21–23]. Thus, it is plausible that data selection and analysis methods also play an important role in MFO and Fat_{max} estimations. There are two known factors that could influence on MFO and Fat_{max} related to data selection and data analysis: (i) the time interval selected to conduct the data analysis (e.g. average of the last 30, 60, 90 or 120 seconds) [5,6,8,13,17,24], and (ii) the data analysis approaches to determine MFO and Fat_{max}, that is the highest measured value, commonly known as measured-values approach [24], the highest point in a polynomial-curve built with the measured values with intersection at (0;0) [6,17,24,25] , the LIPOXmax method [26] or the sine model [24]). Croci et al. (2014) investigated the intra-individual variability of Fat_{max} measurements determined using some of these data analysis approaches. However, they did not consider different time intervals for data analysis (they only used the last 120 s), and their study participants were 15 healthy, moderately trained male volunteers. To our knowledge, there are no studies examining whether the selection of different time intervals and the use the two most used data analysis approaches (i.e. measured-values vs. polynomial-curve) influence MFO and Fat_{max} estimations in sedentary adults of both sexes.

Considering MFO and Fat_{max} as important factors related to metabolic diseases that became increasingly popular during the last years, it is of importance to determine which methodological procedures are better to get a valid and reliable MFO and Fat_{max} estimation. We analyzed the impact of using different pre-defined time intervals on MFO and Fat_{max} estimations. Moreover, we also examined the impact of applying two different data analysis approaches (measured-values vs. polynomial-curve) on MFO and Fat_{max} estimations in sedentary adults. We hypothesised that the use of different pre-defined time intervals may affect the MFO and Fat_{max} estimations independently of the data analysis approach, due to the fact that sedentary individuals likely need longer time to reach a stable measurement when different intensities are applied.

MATERIAL & METHODS

Participants

A total of 151 (97 women) sedentary adults aged 29.2 ± 13.2 years old participated in the current study. Participants were enrolled in the ACTIBATE study (n=109, 74 women, aged 22.1 ± 2.2 years old) [27] (ClinicalTrials.gov. ID:NCT02365129) or in the FIT-AGEING study (n=42, 23 women, aged 52.1 ± 4.2 years old) [28] (Clinicaltrial.gov. ID: NCT03334357). They reported being: (i) sedentary, (ii) non-smokers, (iii) not taking any medication, (iv) not having acute or chronic illness, and (v) not being pregnant. Both studies were approved by the Human Research Ethics Committee of the University of Granada (nº 924), and by the Human Research Ethics Committee of the Junta de Andalucía (nº 0838-N-2017), and followed the revised ethical guidelines of the Declaration of Helsinki (last revision). All participants signed the written informed consent before their enrolment.

Study design

The current study followed a single-center, cross-sectional design, and was conducted between September-November 2016, and September-December 2017. Participants were asked to avoid any moderate or vigorous physical activity (24 and 48 hours, respectively) before the testing day, and not to consume dietary supplements and/or stimulant beverages during the 24 hours before to test. Participants came to the research center in a fasting state of 6-7 h (6.2±0.5h) and arrived at the laboratory with a minimum of physical activity.

Weight and height were measured on a separate day at 8:15 a.m., after a pre-established dinner, in a fasting condition (at least 12 h, except for water intake), without shoes, and with minimal clothing, using a digital integrating scale and stadiometer (Seca 760, Electronic Column Scale, Hamburg, Germany). BMI was calculated as weight (kg)/height (m^2), and body composition (LM and FM) was determined by a Dual Energy X-ray Absorptiometry (HOLOGIC, Discovery Wi) scan. LMI and FMI were calculated as LM (kg)/height (m^2), and FM (kg)/height (m^2), respectively.

Graded exercise protocol

The protocol began with a maximal walking speed test on a treadmill (H/P/cosmos pulsar, H/P/cosmos sports & medical GmbH, Nussdorf-Traunstein, Germany) which started with 30 seconds at 4 km/h (gradient of 0%) followed by 45 seconds at 5.5 km/h. After that, the speed was increased by 1 km/h every 45 seconds until the maximal walking speed was reached (see Figure 1A). Then, after approximately 3 minutes resting, a graded exercise protocol was performed on the treadmill to determine MFO and Fat_{max} (see Figure 1). The test started with 3 minutes warm-up at 3.5 km/h (gradient 0%), and the speed was increased by 1 km/h every 3 minutes until the maximal walking speed was reached. Thereafter, the treadmill speed was kept constant (i.e. maximal walking speed) with the gradient increasing by 2% every 3 minutes until the RER was ≥ 1.0 [29]. The protocol finished with 5 minutes of recovery walking at 4 km/h (gradient 0%). The protocol duration was ~60 min, including rest, maximal walking speed protocol, graded exercise protocol, and recovery (see Figure 1A). Respiratory gas exchange was collected during the whole test through IC (CPX Ultima CardiO2, Medical Graphics Corp, St Paul, USA). A face-mask (model 7400, Hans Rudolph Inc, Kansas City, MO, USA), equipped with a prevent™ metabolic flow sensor (Medgraphics Corp, Minnesota, USA) was used for gases collection.

We determined VO_2max in a separate day (separated by 3-14 days) using a maximum treadmill exercise test following the modified Balke protocol [30]. In this test, the obtained gas exchange parameters were averaged every 5 seconds using the Breeze Suite software. The criteria for achieving VO_2max were: RER ≥ 1.1, a plateau in VO_2 (change of <100 ml/min in the last 3 consecutives 10 seconds stage), and a heart rate within 10 beats/min of the age-predicted maximal heart rate (209–0.73 ∗ age) [31]. We considered the peak oxygen uptake value during the maximum treadmill exercise test when these criteria were not met [31].

Gases processing and analysis

Obtained gas exchange parameters in the MFO and Fat_{max} test were averaged every 10 seconds with the Breeze Suite software (version 8.1.0.54 SP7, MGC Diagnostic®). A total of 13-time intervals (see Figure 1B) were pre-defined and applied in all the 3 minutes stages: (i) First 30 seconds mean; (ii) Second 30 seconds mean; (iii) Third 30 seconds mean; (iv) Fourth 30 seconds mean; (v) Fifth 30 seconds mean; (vi) Last 30 seconds mean; (vii) First 60 seconds mean; (viii) Middle 60 seconds mean; (ix) Last 60 seconds mean; (x) First 90 seconds mean; (xi) Last 90 seconds

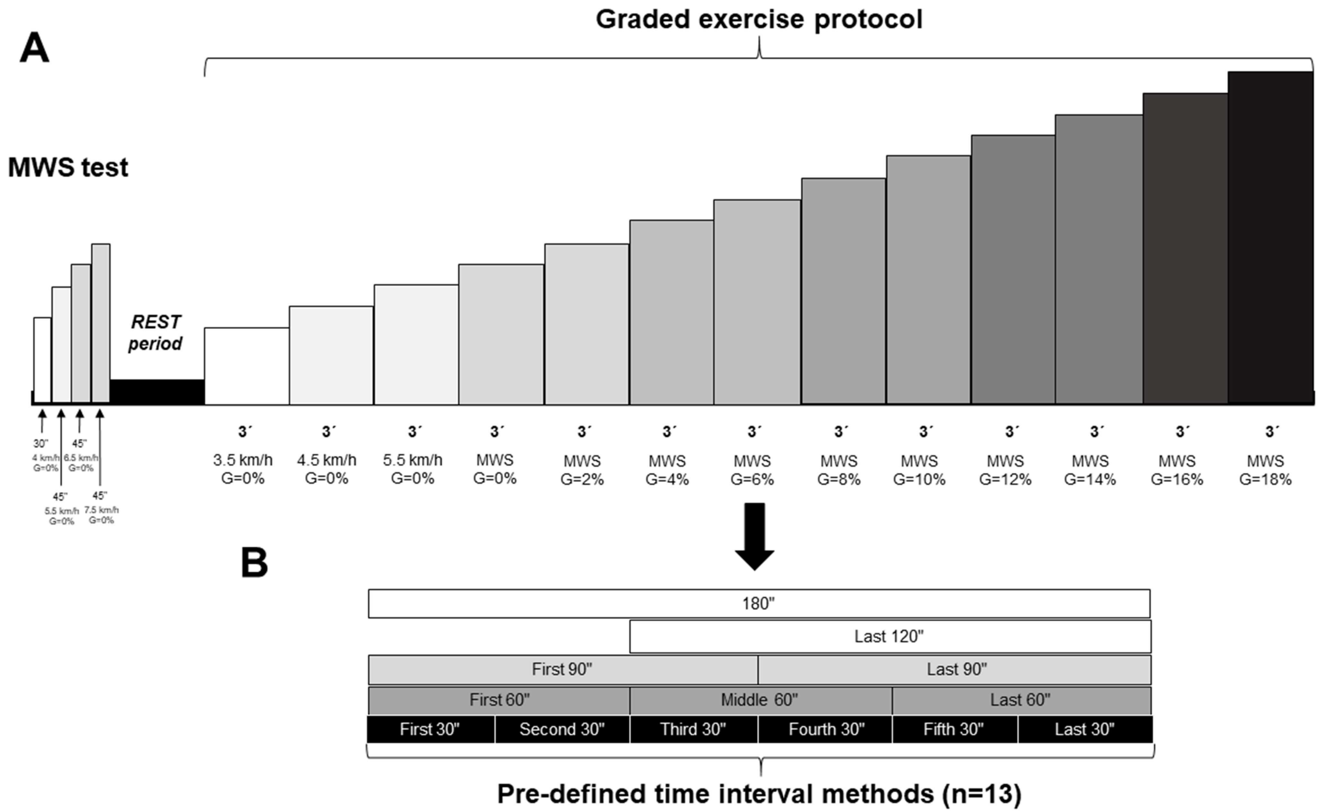

Figure 1. Maximal walking speed protocol, graded exercise test protocol to determine maximal fat oxidation and the exercise intensity that elicit the maximal fat oxidation (Panel A), and the pre-defined time intervals (n=13) applying 3 minutes stages in the graded exercise protocol (Panel B). Abbreviations: G; gradient, MWS; Maximal Walking Speed.

mean; and (xii) Last 120 seconds mean; and (xiii) 180 seconds mean.

Fat oxidation rates were calculated using the Frayn stoichiometric equations with the assumption that urinary nitrogen excretion was 0 [32]. We determined MFO and Fat_{max} using 2 different data analysis approaches: (i) measured-values: we obtained MFO and Fat_{max} data from the stage at which fat oxidation rate was higher, and the corresponding intensity was selected [24]; (ii) Polynomial-curve: we calculated MFO and Fat_{max} constructing a third polynomial curve with intersection at (0;0) from a graphical depiction of fat oxidation values as a function of exercise intensity [24].

Statistical analysis

The Shapiro-Wilk test, visual check of histograms, Q-Q, and box plots were used to verify the normal distribution of all variables. The descriptive parameters are reported as mean and SD. We conducted repeated measures ANOVA with Bonferroni correction to compare MFO and Fat_{max} across the pre-defined time intervals in the graded exercise protocol, using measured-values and the polynomial-curve data analysis approaches separately. The main analyses were conducted with the most used time intervals (i.e. last 30, 60, 90 and 120 seconds). We conducted a paired t-test to study the differences on MFO and Fat_{max} between measured-values and the polynomial-curve data analysis approaches. In order to understand whether the cardiorespiratory fitness level (i.e. VO_2max) influences the MFO and Fat_{max} calculated by different time intervals methods and using different data analysis approaches, we performed a repeated measures ANOVA to compare MFO and Fat_{max} across the pre-defined time intervals using measured-values vs. polynomial-curve data analysis approaches after dividing our participants into tertiles based on VO_2max (high VO_2max vs. low VO_2max). Finally, we conducted a repeated measures ANOVA to compare MFO and Fat_{max} measurements calculated by different stoichiometric equations across the time intervals. The analyses were conducted using the Statistical Package for Social Sciences (IBM Corporation, Chicago, IL, USA), and the level of significance was set at <0.05.

RESULTS

The characteristics of the study sample are shown in Table 1.

Figure 2 shows the MFO and Fat_{max} measurements across the time intervals (i.e. last 30, 60, 90 and 120 seconds) applying 2 different data analysis approaches (measured-values and the polynomial-curve). The MFO (expressed in absolute term and relative to the LM) and Fat_{max} data of the other pre-defined time intervals methods can be seen in Figure 3.

Table 1. Descriptive characteristic of participants.

	All (n=151)		Men (n=54)		Women (n=97)	
Age (years)	29.7	(13.4)	32.0	(14.5)	28.3	(12.6)
BMI (kg/m²)	25.7	(4.7)	28.1	(5.1)	24.5	(3.9)
LM (kg)	42.3	(10.2)	53.6	(6.7)	36.1	(5.2)
LMI (kg/m²)	14.9	(2.6)	17.4	(2.1)	13.4	(1.6)
FM (%)	37.4	(8.0)	32.7	(7.8)	40.0	(7.0)
FMI (kg/m²)	9.6	(3.3)	9.3	(3.6)	9.8	(3.1)
VO_2max (ml/kg/min)	37.4	(8.9)	38.5	(10.1)	36.8	(8.2)
MWS (%)						
5.5 km/h	84	(55.6)	20	(37.0)	64	(66.0)
6.5 km/h	61	(40.4)	29	(53.7)	32	(33.0)
7.5 km/h	6	(4.0)	5	(9.3)	1	(1.0)

Data are presented as mean (standard deviation), except for MWS that is expressed as n (%). Abbreviations: MWS: Maximal Walking Speed

There were significant differences in MFO in absolute and relative terms across time intervals applying the measured-values data analysis approach (range: from 0.34 to 0.35 g/min ; P=0.001; Figure 2A, and range: from 0.0077 to 0.0078 g/min; P=0.002; Figure 2C), while no differences were obtained in MFO across time intervals when applying the polynomial-curve data analysis approach (range: from 0.36 to 0.37 g/min; P≥0.077; Figure 2B and range: from 0.0083 to 0.00784 g/min ; P=0.102; Figure 2D. There were no differences in Fat_{max} across time intervals applying both measured-values and polynomial-curve data analysis approaches (range: from 43.1 to 45.9 % of VO_2max; P=0.797; Figure 2E, and range: from 46.3 to 47.2 % of VO_2max; P=0.781; Figure 2F, respectively). The statistical analysis of the results remained unchanged after adjusting for sex, BMI, LMI, and FMI (all P≥0.2).

We also conducted a comparison between MFO (expressed in absolute term and relative to LM) and Fat_{max} calculated with the measured-values and the polynomial-curve data analysis approaches across time intervals (see Table 2). There were significant differences in MFO when it was calculated by measured-values or by polynomial-curve across time intervals in the whole sample (mean differences range from 6.69 to 7.29 %, all P≤0.002 for MFO expressed in absolute terms, and mean differences range from 6.69 to 9.09 %, all P≤0.003 for MFO expressed relative to the LM). On the other hand, Fat_{max} did not differ between approaches (mean differences range from 0.41 to 1.55 %, all P≥0.2).

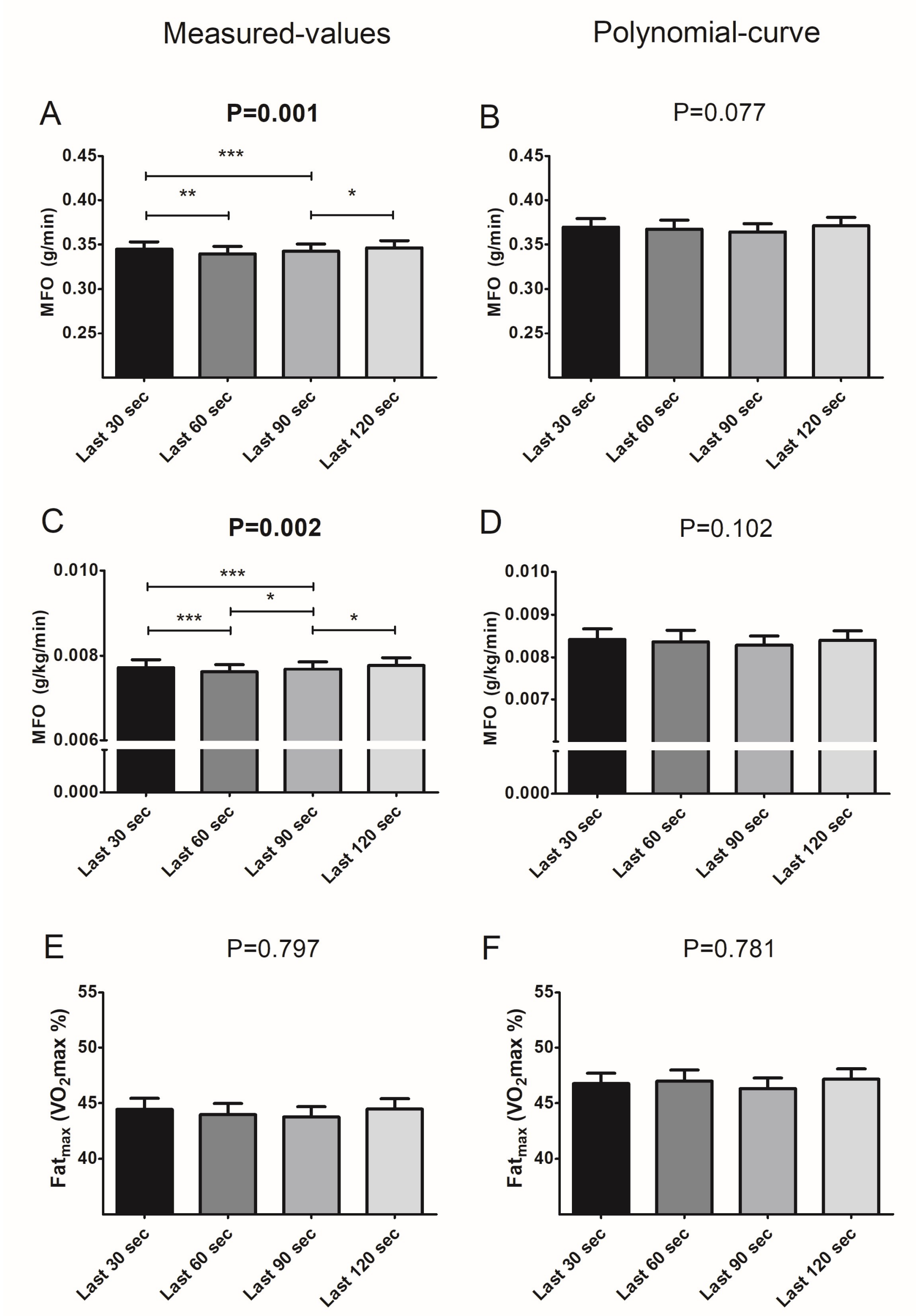

Figure 2. Maximal fat oxidation (MFO), and exercise intensity that elicits MFO (Fat_{max}) measurements across different time interval, using 2 different data analysis approaches: MFO by measured values data analysis approach (Figure 2A and 2C), MFO by polynomial-curve data analysis approach (Figure 2B and 2D), Fat_{max} by measured values data analysis approach (Figure 2E), and Fatmax by polynomial-curve data analysis approach (Figure F). P value from repeated measures ANOVA (*P<0.05; **P<0.01; ***P<0.001).

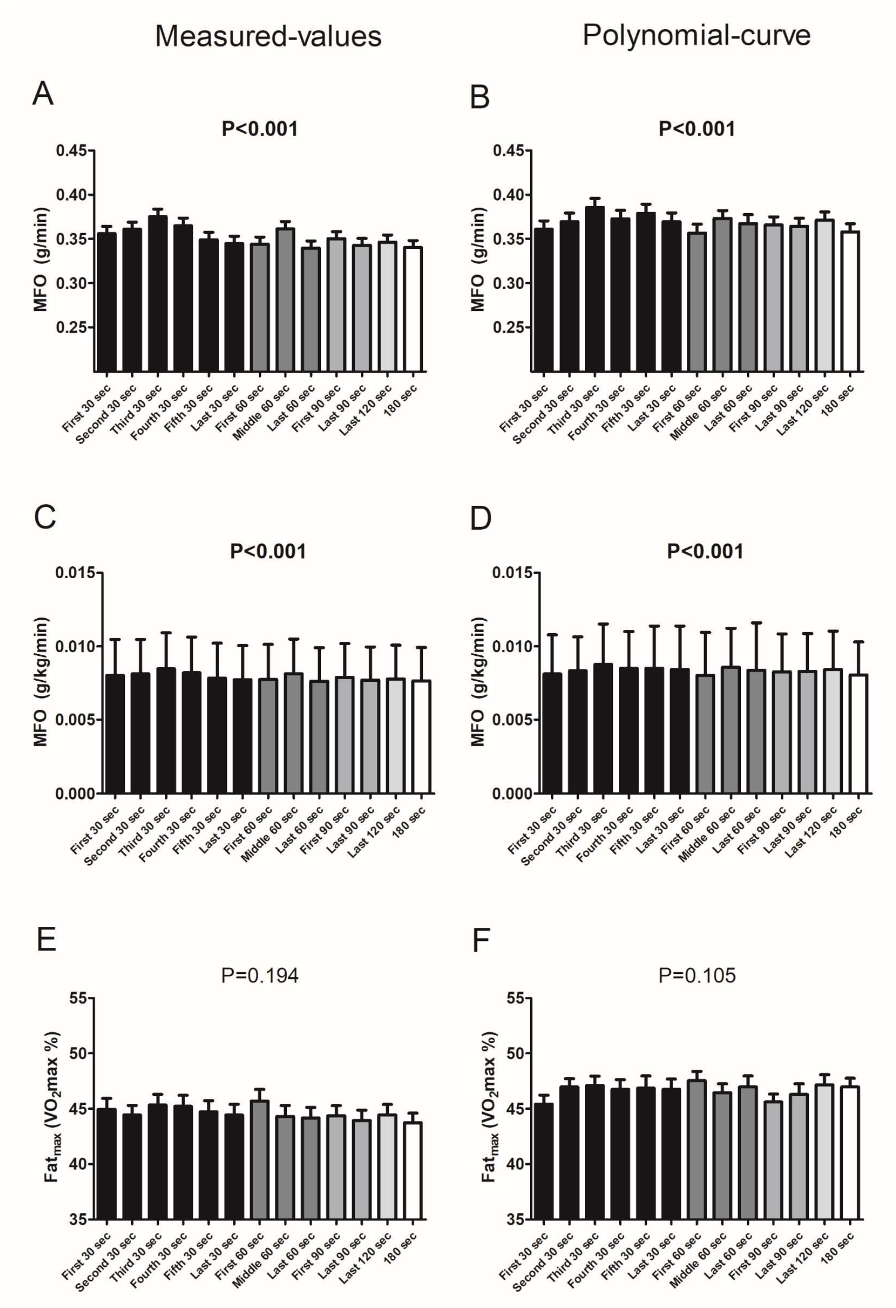

Figure 3. Maximal fat oxidation (MFO), and exercise intensity that elicits MFO (Fat_{max}) measurements across different time interval, using 2 different data analysis approaches: MFO by measured values data analysis approach (Figure 2A and 2C), MFO by polynomial-curve data analysis approach (Figure 2B and 2D), Fatmax by measured values data analysis approach (Figure 2E), and Fat_{max} by polynomial-curve data analysis approach (Figure F). Black columns represent time intervals of 30 seconds; dark grey columns represent time intervals of 60 seconds; grey columns represent time intervals of 90 seconds; light grey columns represent time intervals of 120 seconds; and white columns represent time intervals of 180 seconds. P value from repeated measures ANOVA.

Table 2. Maximal fat oxidation (MFO), and exercise intensity that elicits MFO (Fat_{max}) measurements across different time intervals using 2 different data analysis approaches: measured-values vs. polynomial-curve data analysis approaches.

	Measured-values		Polynomial-curve		Δ (%)	P	Cohen´s D
Last 30 sec							
MFO (g/min)	0.34	(0.11)	0.37	(0.12)	7.20	**0.002**	0.26
MFO (g/kg/min)	0.0078	(0.0022)	0.0084	(0.0026)	7.69	**0.001**	0.25
Fat_{max} (VO_2max%)	47.32	(11.99)	46.75	(9.17)	1.21	0.520	0.06
Last 60 sec							
MFO (g/min)	0.34	(0.10)	0.37	(0.12)	7.29	**0.002**	0.27
MFO (g/kg/min)	0.0077	(0.0022)	0.0083	(0.0022)	7.79	**0.001**	0.27
Fat_{max} (VO_2max%)	46.24	(11.64)	46.96	(10.46)	1.55	0.278	0.07
Last 90 sec							
MFO (g/min)	0.34	(0.10)	0.36	(0.11)	6.69	**0.001**	0.26
MFO (g/kg/min)	0.0077	(0.0021)	0.0084	(0.0023)	9.09	**0.003**	0.32
Fat_{max} (VO_2max%)	45.86	(10.71)	46.30	(9.77)	0.97	0.387	0.04
Last 120 sec							
MFO (g/min)	0.35	(0.10)	0.37	(0.12)	6.97	**0.001**	0.23
MFO (g/kg/min)	0.0078	(0.0024)	0.0084	(0.0020)	7.69	**0.002**	0.27
Fat_{max} (VO_2max%)	46.97	(10.99)	47.16	(9.86)	0.41	0.725	0.01

Data are presented as mean (SD). Paired-sample t-test were used to examine the differences between MFO and Fat_{max} calculated by 2 different data analyses approaches (measured-values vs. polynomial-curve) across different time intervals. Statistically significant values are shown in bold ($P \leq 0.05$). Abbreviations: VO_2max: maximal oxygen uptake from the maximum treadmill exercise test.

Figure 4 shows MFO and Fat_{max} across time intervals using measured-values and the polynomial-curve data analysis approaches in individuals with high and low VO_2max. There were no differences in MFO and Fat_{max} across time intervals in both data analysis approaches, comparing high and low VO_2max individuals (all $P \geq 0.267$, see Figure 4).

Finally, we compared MFO and Fat_{max} measurements calculated by 6 different stoichiometric equations (i.e. Frayn, Jequier et al., Péronnet & Massicotte, Brouwer, Elia & Livesey, and Jeaukendrup & Achten [29,32–36]) across the time intervals using both measured-values and the polynomial-curve data analysis approaches. There were no significant differences in MFO and Fat_{max} in all cases (data not shown).

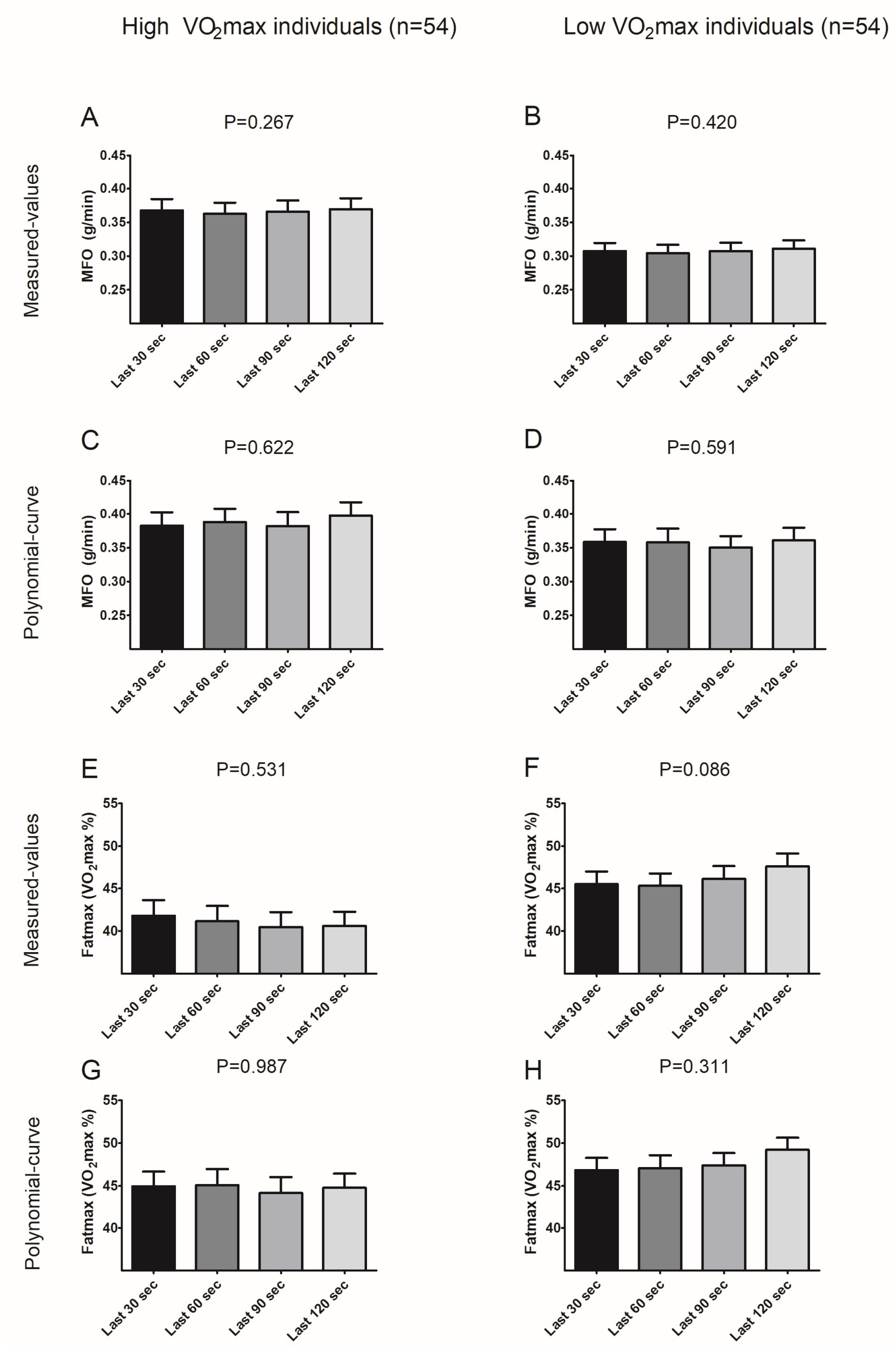

Figure 4. Maximal fat oxidation (MFO), and exercise intensity that elicits MFO (Fat_{max}) measurements across different time interval, dividing the participants in terciles by maximal oxygen consumption (VO_2max), and using 2 different data analysis approaches: MFO by measured values data analysis approach (Figure 3A, and 3B), MFO by polynomial-curve data analysis approach (Figure 3C, and 3D), Fatmax by measured values data analysis approach (Figure 3E, and 3F), and Fat_{max} by polynomial-curve data analysis approach (Figure 3G, and 3H). P value from repeated measures ANOVA.

DISCUSSION

The primary findings indicate that there are no differences in MFO and Fat_{max} across the selection of different time intervals in the last part of the stage (i.e. last 30, 60, 90- and 120-seconds time intervals) using the polynomial-curve data analysis. However, we observed significant differences in MFO estimation using the measured-values data analysis approach, although a small effect size was obtained when we compared MFO and Fat_{max} estimations by measured-values vs. polynomial-curve data analysis approaches across different time intervals. Importantly, significant differences were noted in MFO, but not in Fat_{max}, estimations using measured-values vs. polynomial-curve data analysis approaches. Therefore, the use of polynomial-curve data analysis approach leads to similarly estimates of MFO and Fat_{max} independently of the time interval used in sedentary adults.

It is widely accepted that data selection and analysis have an important influence on BMR and meal-induced thermogenesis estimations by IC using breath-by-breath metabolic carts [21–23], but data are scarce about the impact of methods for data analysis on MFO and Fat_{max}. It is widely recognized that there is a high variability in MFO and Fat_{max} between individuals, which can be attributed to numerous factors such as sex, training status, nutritional status or exercise modality [9]. We observed that the use of different time intervals for data analysis and different data analysis approaches influence the estimation of MFO and Fat_{max}, concluding that the use of the polynomial-curve data analysis approach, whenever the last seconds (i.e. last 30, 60, 90- and 120-seconds time intervals) of the stage are used, is a method that allow to reach a stable measurement and, consequently, to well estimate MFO and Fat_{max}. These findings can be explained because the polynomial-curve data analysis approach may suppose a better mathematical model than measured-values data analysis approach, and the use of time intervals that include the last seconds of the stage implies that the probability of reach a stable measurement is higher than whether they include the first second of the stage. Indeed, significant differences in MFO were observed when compared all time intervals selected in our study independently of the Fat_{max} data analysis approach applied, while no differences were noted when compared time intervals that include the last seconds of the stage applying the polynomial-curve data analysis approach.

MFO and Fat_{max} values of different studies have been compared without considering the time interval selected for data analysis [5,6,8,13,17,24]. Our results clearly indicate that the comparison between MFO obtained by the different time intervals methods (last 30 seconds, last 60 seconds, last 90 seconds, and last 120 seconds) applying measured-values data analysis approach must be considered cautiously, since significant differences were observed across time intervals. However, no significant differences were obtained neither in Fat_{max} using measured-values analysis approach, nor in MFO and Fat_{max} applying measured-values vs. polynomial-curve data analysis approaches.

Our results support the notion that training status strongly influences MFO, whereas the differences in Fat_{max} are not still clearly established [9,11,12,37,38]. High VO_2max individuals showed greater MFO compared with low VO_2max individuals (0.34 vs. 0.30 g/min; P=0.005), whereas no differences were observed in Fat_{max} (45.0 vs. 46.8 %; P=0.103), which concur with previous studies [9,11,12,37,38]. Our findings suggest that the results obtained in our study persist when different time intervals and Fat_{max} data analysis approaches were compared in sedentary adults. Further studies are needed to elucidate, whether these results remain when different data selection and analysis are compared in trained individuals and/or patients.

It has been suggested that inter-study discrepancies in MFO and Fat_{max} can be attributed to the stoichiometric equation used to calculate substrate oxidization applied to calculate fuel oxidation [14,17–20]. Although previous studies proposed that the use of different stochiometric equations hamper the inter-study comparison [9,39], our results revealed that no differences were observed in MFO and Fat_{max} across different time intervals applying 2 different data analysis approaches (measured-values vs. polynomial-curve) in sedentary adults.

Some studies have previously mentioned that it is necessary to reach a stable measurement in the respiratory parameter values during each graded exercise protocol stage to get a valid measure of MFO and Fat_{max} [40]. Considering that we obtained a narrow CV in the respiratory parameters, we can conclude that a stable measurement was reached in each time interval selected.

Limitations

Our study has some limitations: (i) We do not know whether our findings apply when MFO and Fat_{max} are calculated by a cycle ergometer graded exercise protocol, or in physically active individuals. (ii) We averaged gas exchange each 10 seconds and, consequently, we did not have available a large amount of data for some cases. (iii) We applied a 3 minutes duration for each stage based on previous studies [6,7,13,41]. However, fat and carbohydrate oxidation values obtained by 3 minutes stage duration are different than those obtained by 6 minutes stage duration in very sedentary patients, showing a substantial underestimation of fat oxidation rates [42]. This fact could have influenced our results. (iv) The work rates of our test protocol were based on the treadmill grade, instead of a metabolically-derived marker (i.e. % of VO_2max).

CONCLUSION

In conclusion, our results revealed that there are no differences in MFO and Fat_{max} across the selection of different time intervals in the last part of the intensity stages using the polynomial-curve data analysis approach. We observed significant differences in MFO between measured-values vs. polynomial-curve data analysis approaches in all the studied time intervals. Therefore, based on our data, the method of choice for estimating MFO and Fat_{max} should be the polynomial-curve data analysis approach, while the time interval selected (if it contains the last seconds of the stage) and the stoichiometric equation do not impact on MFO and Fat_{max} estimations. Future studies should consider these methodological aspects when analysing MFO and Fat_{max}

REFERENCES

1 Kelley DE, Goodpaster B, Wing RR, Simoneau JA. Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. *Am J Physiol* 1999; **277**: E1130-41.

1 Kelley DE, Goodpaster B, Wing RR, Simoneau JA. Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. *Am J Physiol* 1999; **277**: E1130-41.

2 Pérez-Martin A, Dumortier M, Raynaud E, Brun JF, Fédou C, Bringer J *et al.* Balance of substrate oxidation during submaximal exercise in lean and obese people. *Diabetes Metab* 2001; **27**: 466–74.

3 Rosenkilde M, Nordby P, Nielsen LB, Stallknecht BM, Helge JW. Fat oxidation at rest predicts peak fat oxidation during exercise and metabolic phenotype in overweight men. *Int J Obes (Lond)* 2010; **34**: 871–7.

4 Aucouturier J, Rance M, Meyer M, Isacco L, Thivel D, Fellmann N *et al.* Determination of the maximal fat oxidation point in obese children and adolescents: Validity of methods to assess maximal aerobic power. *Eur J Appl Physiol* 2009; **105**: 325–331.

5 Chu L, Riddell MC, Takken T, Timmons BW. Carbohydrate intake reduces fat oxidation during exercise in obese boys. *Eur J Appl Physiol* 2011; **111**: 3135–41.

6 Dandanell S, Husted K, Amdisen S, Vigelsø A, Dela F, Larsen S *et al.* Influence of maximal fat oxidation on long-term weight loss maintenance in humans. *J Appl Physiol* 2017; **123**: 267–274.

7 Dandanell S, Præst CB, Søndergård SD, Skovborg C, Dela F, Larsen S *et al.* Determination of the exercise intensity that elicits maximal fat oxidation in individuals with obesity. *Appl Physiol Nutr Metab* 2017; **42**: 405–412.

8 Mogensen M, Vind BF, Højlund K, Beck-Nielsen H, Sahlin K. Maximal lipid oxidation in patients with type 2 diabetes is normal and shows an adequate increase in response to aerobic training. *Diabetes, Obes Metab* 2009; **11**: 874–883.

9 Maunder E, Plews DJ, Kilding AE. Contextualising Maximal Fat Oxidation During Exercise: Determinants and Normative Values. *Front Physiol* 2018; **9**: 1–13.

10 Achten J, Gleeson M, Jeukendrup AE. Determination of the exercise intensity that elicits maximal fat oxidation. *Med Sci Sports Exerc* 2002; **34**: 92–7.

11 Venables MC, Achten J, Jeukendrup AE. Determinants of fat oxidation during exercise in healthy men and women: a cross-sectional study. *J Appl Physiol* 2005; **98**: 160–167.

12 Fletcher G, Eves FF, Glover EI, Robinson SL, Vernooij CA, Thompson JL *et al.* Dietary intake is independently associated with the maximal capacity for fat oxidation during exercise. *Am J Clin Nutr* 2017; **105**: 864–872.

13 Randell RK, Rollo I, Roberts TJ, Dalrymple

KJ, Jeukendrup AE, Carter JM. Maximal Fat Oxidation Rates in an Athletic Population. *Med Sci Sports Exerc* 2017; **49**: 133–140.

14 Mohebbi H, Nourshahi M, Ghasemikaram M, Safarimosavi S. Effects of exercise at individual anaerobic threshold and maximal fat oxidation intensities on plasma levels of nesfatin-1 and metabolic health biomarkers. *J Physiol Biochem* 2015; **71**: 79–88.

15 Rosenkilde M, Reichkendler MH, Auerbach P, Bonne TC, Sjödin A, Ploug T *et al.* Changes in peak fat oxidation in response to different doses of endurance training. *Scand J Med Sci Sport* 2015; **25**: 41–52.

16 Mendelson M, Jinwala K, Wuyam B, Levy P, Flore P. Can crossover and maximal fat oxidation rate points be used equally for ergocycling and walking/running on a track? *Diabetes Metab* 2012; **38**: 264–270.

17 Andersson-Hall U, Pettersson S, Edin F, Pedersen A, Malmodin D, Madsen K. Metabolism and whole-body fat oxidation following postexercise carbohydrate or protein intake. *Int J Sport Nutr Exerc Metab* 2016; **28**: 37–45.

18 Gutiérrez-Hellín J, Del Coso J. Dose–Response Effects of p-Synephrine on Fat Oxidation Rate During Exercise of Increasing Intensity. *Phyther Res* 2018; **32**: 370–374.

19 De Souza Silveira R, Carlsohn A, Langen G, Mayer F, Scharhag-Rosenberger F. Reliability and day-to-day variability of peak fat oxidation during treadmill ergometry. *J Int Soc Sports Nutr* 2016; **13**: 1–7.

20 Peric R, Meucci M, Bourdon PC, Nikolovski Z. Does the aerobic threshold correlate with the maximal fat oxidation rate in short stage treadmill tests? *J Sports Med Phys Fitness* 2017.

21 Alcantara JMA, Sanchez-Delgado G, Martinez-Tellez B, Merchan-Ramirez E, Labayen I, Ruiz JR. Congruent validity and inter-day reliability of two breath by breath metabolic carts to measure resting metabolic rate in young adults. *Nutr Metab Cardiovasc Dis* 2018; **28**: 929–936.

22 Fullmer, S, Benson-Davies, S, Earthman, CP, Frankenfield, DC, Gradwell, E, Lee, P, Trabulsi J. Evidence analysis library review of best practices for performing indirect calorimetry in healthy and Non-critically ill individuals. *J Acad Nutr Diet* 2015; **115**: 1417–1446.

23 Sanchez-Delgado G, Alcantara JMA, Ortiz-Alvarez L, Xu H, Martinez-Tellez B, Labayen I *et al.* Reliability of resting metabolic rate measurements in young adults: Impact of methods for data analysis. *Clin Nutr* 2017.

24 Croci I, Borrani F, Byrne NM, Byrne N, Wood RE, Wood R *et al.* Reproducibility of Fatmax and fat oxidation rates during exercise in recreationally trained males. *PLoS One* 2014; **9**: e97930.

25 Chenevière X, Malatesta D, Gojanovic B, Borrani F. Differences in whole-body fat oxidation kinetics between cycling and

running. *Eur J Appl Physiol* 2010; **109**: 1037–45.

26 Besnier F, Lenclume V, Gérardin P, Fianu A, Martinez J, Naty N *et al*. Individualized exercise training at maximal fat oxidation combined with fruit and vegetable-rich diet in overweight or obese women: The LIPOXmax-réunion randomized controlled trial. *PLoS One* 2015; **10**: 27–40.

27 Sanchez-Delgado G, Martinez-Tellez B, Olza J, Aguilera CM, Labayen I, Ortega FB *et al.* Activating brown adipose tissue through exercise (ACTIBATE) in young adults: Rationale, design and methodology. *Contemp Clin Trials* 2015; **45**: 416–425.

28 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.

29 Jeukendrup AE, Wallis GA. Measurement of substrate oxidation during exercise by means of gas exchange measurements. *Int J Sports Med* 2005; **26 Suppl 1**: S28-37.

30 Balke B, Ware RW. An experimental study of physical fitness of Air Force personnel. *U S Armed Forces Med J* 1959; **10**: 675–88.

31 Midgley AW, McNaughton LR, Polman R, Marchant D. Criteria for determination of maximal oxygen uptake: A brief critique and recommendations for future research. *Sport Med* 2007; **37**: 1019–1028.

32 Frayn KN. Calculation of substrate oxidation rates in vivo from gaseous exchange. *J Appl Physiol* 1983; **55**: 628–34.

33 Brouwer E. On simple formulae for calculating the heat expenditure and the quantities of carbohydrate and fat oxidized in metabolism of men and animals, from gaseous exchange (Oxygen intake and carbonic acid output) and urine-N. *Acta Physiol Pharmacol Neerl* 1957; **6**: 795–802.

34 Elia M, Livesey G. Energy expenditure and fuel selection in biological systems: the theory and practice of calculations based on indirect calorimetry and tracer methods. *World Rev Nutr Diet* 1992; **70**: 68–131.

35 Jéquier E, Acheson K, Schutz Y. Assessment of energy expenditure and fuel utilization in man. *Annu Rev Nutr* 1987; **7**: 187–208.

36 Péronnet F, Massicotte D. Table of nonprotein respiratory quotient: an update. *Can J Sport Sci* 1991; **16**: 23–9.

37 Purdom T, Kravitz L, Dokladny K, Mermier C. Understanding the factors that effect maximal fat oxidation. *J Int Soc Sports Nutr* 2018; **15**: 1–10.

38 Nordby P, Saltin B, Helge JW. Whole-body fat oxidation determined by graded exercise and indirect calorimetry: a role for muscle oxidative capacity? *Scand J Med Sci Sports* 2006; **16**: 209–14.

39 Bircher S, Knechtle B. Relationship between Fat Oxidation and Lactate Threshold in Athletes and Obese Women

and Men. *J Sports Sci Med* 2004; **3**: 174–81.

40 Dandanell S, Præst CB, Søndergård SD, Skovborg C, Dela F, Larsen S *et al.* Determination of the exercise intensity that elicits maximal fat oxidation in individuals with obesity. *Appl Physiol Nutr Metab* 2017; **42**: 405–412.

41 Fletcher G, Eves FF, Glover EI, Robinson SL, Vernooij CA, Thompson JL *et al.* Dietary intake is independently associated with the maximal capacity for fat oxidation during exercise. *Am J Clin Nutr* 2017; **105**: 864–872.

42 Bordenave S, Flavier S, Fédou C, Brun JF, Mercier J. Exercise calorimetry in sedentary patients: procedures based on short 3 min steps underestimate carbohydrate oxidation and overestimate lipid oxidation. *Diabetes Metab* 2007; **33**: 379–84.

Optimizing maximal fat oxidation assessment by a graded exercise protocol when the test should be ended
(Study 5)

ABSTRACT

MFO and Fat$_{max}$ are considered important factors related to metabolic health and performance. Numerous MFO and Fat$_{max}$ data collection and analysis approaches have been applied, which may have influenced their estimation during an incremental graded exercise protocol. Despite the heterogeneity of protocols used, all studies consistently stopped the MFO and Fat$_{max}$ test when the REr was 1.0. It remains unknown however whether reaching a RER of 1.0 is required to have an accurate, reliable and valid measure of MFO and Fat$_{max}$. We aimed to investigate the RER at which MFO and Fat$_{max}$ occurred in sedentary and trained healthy adults. A total of 182 sedentary aged between 18-65 years participated in the study. MFO and Fatmax were calculated by an incremental graded exercise protocol before and after 2 exercise-based intervention. Our findings suggest that a graded exercise protocol aiming to determine MFO and Fat$_{max}$ could end when a RER=0.93 is reached in sedentary healthy adults, and when a RER=0.90 is reached in trained adults independently of sex, age, body weight status, or the Fatmax data analysis approach. In conclusion, we suggest reducing the RER from 1.0 to 0.95 to be sure that MFO is reached in outliers individuals. This methodological consideration has important clinical implications, since it would allow to apply smaller workload increments and/or to extend the stage duration to attain the steady-state, without increasing the test duration.

BACKGROUND

Metabolic flexibility is defined as the capacity of an individual to respond or adapt the nutrients balance to different metabolic demands [1]. This concept has been particularly studied in the fasted state as well as in the shift from fasting to fed [2]. MFO and Fat_{max} are key factors of metabolic flexibility [1,3], affecting both endurance performance and metabolic health [1,3], therefore their accurate determination is of clinical interest.

In 2002, Achten et al. [4] were the first to validate a graded exercise protocol to determine MFO and Fat_{max} using a 3-min duration stages and 35-W workload increments. Later, other graded exercise protocols to determine MFO and Fat_{max} have been applied considering participants' sex, age, training status, or weight status [5]. Two specific issues have traditionally been modified on the graded exercise protocol: (i) the stage duration (e.g. from 1-min to 10-min), and (ii) the workload increment (e.g. from 10-W to 50-W) [5].

There is no consensus regarding the ideal stage duration of a graded exercise protocol for determining MFO and Fat_{max} [5], yet reaching the steady-state seems mandatory [6,7]. Achten et al. [4] showed no differences in MFO and Fat_{max} between 3-min and 5-min stage protocols in moderately trained men [4], whereas others reported that 3-min stage duration is not long enough to reach a steady-state in obese and diabetic patients with very low VO_2max levels, and recommended 6-min stage duration in sedentary patients [8,9].

The workload increment has also largely varied across studies and, alike the stage duration, it has been adjusted to the participant's biological characteristics [3,5]. Applying relatively small workload increments allows to accurately determine MFO and Fat_{max}, independently of the participant´s biological characteristics.

Taking into account the above-mentioned issues, it would be advisable to apply long stage duration to reach the steady-state (e.g. 6-min stage duration for sedentary patients with low levels of VO_2max), and also to select small workload increments (e.g. 10-W increments) to accurately determine MFO and Fat_{max} through a graded exercise protocol. However, this can result in a very long test duration, which could negatively influence determination of MFO and Fat_{max} due to peripheral and/or central fatigue [10]. Therefore, the development of strategies aiming to decrease the total duration of a graded exercise protocol, while using long enough stage durations and relatively small workload increments, is of clinical relevance.

Of note is that despite the heterogeneity of protocols used, all studies consistently stopped the MFO and Fat_{max} test when the RER was 1.0. This criteria was firstly applied by Achten et al. [4] and all the subsequent studies followed the same criteria. It remains unknown however whether reaching a RER of 1 is required to have an accurate, reliable and valid measure of MFO and Fat_{max}. We aimed to investigate the RER at which MFO and Fat_{max} occurred in sedentary and trained healthy adults.

MATERIAL & METHODS

Participants

One-hundred twenty-five young sedentary adults (age 22.1±2.2 years; BMI 25.0±4.8 kg/m^2; VO_2max 41.2±7.8 ml/kg/min; 84 women/41 men) [11] and 42 sedentary middle-aged adults (age 52.1±4.6 years; BMI 27.8±3.6 kg/m^2; VO_2max 30.4±5.6 ml/kg/min; 23 women/19 men) [12] were included in the current study. Both cohorts performed an exercise-based intervention (24-weeks and 12-weeks, respectively) and a total of 57 young trained adults (age 22.6±2.2 years; BMI 24.3±5.1 kg/m^2; VO_2max 44.3±9.5 ml/kg/min; 39 women/18 men) and 31 middle-aged trained adults (age 52.4±4.6 years; BMI 27.1±3.9 kg/m^2; VO_2max 34.8±6.3 ml/kg/min; 16 women/15 men) finished their respective exercise training programs. Before participating in this study, the participants signed an informed consent form. The investigations were approved by the Human Research Ethics Committee of the University of Granada (nº 924), and by the Human Research Ethics Committee of the Junta de Andalucía (nº 0838-N-2017).

Procedures

We assessed MFO and Fat_{max} through a walking graded exercise protocol [13,14] before and after both exercise training programs. The graded exercise protocol began with a 3-min warm-up at 3.5 km/h with a gradient of 0% followed by increments of the treadmill speed of 1 km/h every 3-min until the maximal walking speed (previously determined) was reached. Afterwards, the treadmill speed was maintained and the treadmill gradient increased 2% every 3-min until RER reached 1.0 [15]. We considered the last 1-min of each 3-min stage [16] to calculate fat oxidation using the Frayn stoichiometric equation [17]. We determined MFO and Fat_{max} using the measured-values data analysis approach (i.e. the highest fat oxidation rate recorded across the graded exercise protocol).

RESULTS

RER at Fat_{max} in sedentary healthy adults

We observed a RER at Fat_{max} of 0.82±0.04 (range: 0.70 to 0.93; Figure 1A), which was similar in men and women (0.83±0.05 vs. 0.82±0.04, respectively, P>0.9), in young and middle-aged adults (0.83±0.05 vs. 0.82±0.05, respectively, P>0.8), and across weight status (0.82±0.03, 0.82±0.05 and 0.83±0.05 for

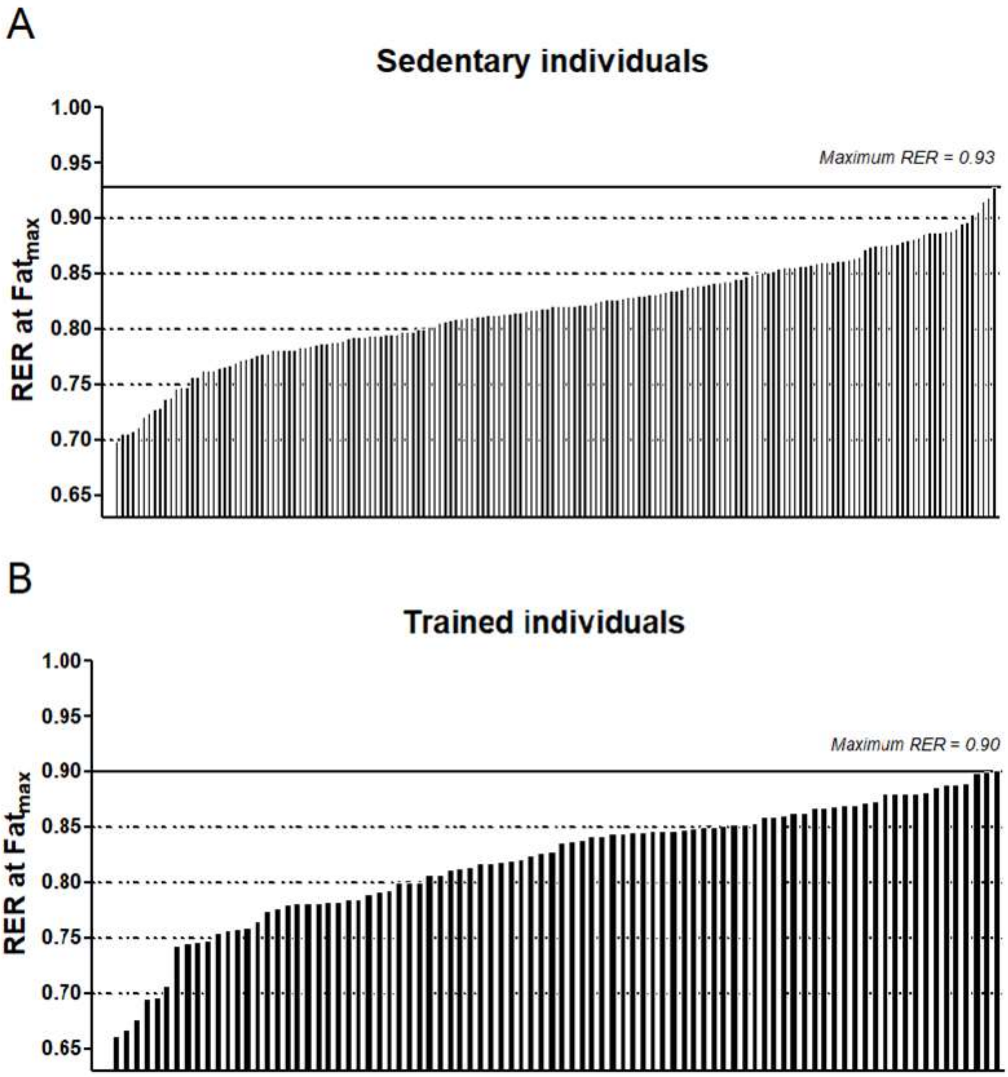

Figure 1. Respiratory exchange ratio (RER) reached at the intensity that elicit the maximal fat oxidation during exercise (Fat_{max}) in: Panel A: 125 young sedentary adults (age 22.1±2.2 years; BMI 25.0±4.8 kg/m²; VO_2max 41.2±7.8 ml/kg/min; 84 women/41 men) and in 42 middle-aged sedentary adults (age 52.1±4.6 years; BMI 27.8±3.6 kg/m²; VO_2max 30.4±5.6 ml/kg/min; 23 women/19 men); and Panel B in 57 young sedentary adults (age 22.6±2.2 years; BMI 24.3±5.1 kg/m²; VO_2max 44.3±9.5 ml/kg/min; 39 women/18 men) and in 31 middle-aged sedentary adults (age 52.4±4.6 years; BMI 27.1±3.9 kg/m²; VO_2max 34.8±6.3 ml/kg/min; 16 women/15 men).

normal-weight, overweight and obese individuals, respectively, P>0.8). Interestingly, the RER at Fat_{max} was between 0.7 and 0.8 in 34.7% (n=58) of participants, between 0.8 and 0.9 in 62.3% (n=104) of participants, and between 0.9 and 0.93 in 3% (n=5) of participants. To note is that the graded exercise protocol total duration was 21.3±4.7 min when RER=1.0, while if the graded exercise protocol had been stopped at the highest registered RER at Fat_{max} (i.e. 0.93), the total duration would have been 13.4±5.3 min (mean difference 7.9±2.9 min).

RER at Fat_{max} in trained healthy adults

The RER at Fat_{max} was 0.82±0.06 (range: 0.67 to 0.90; Figure 1B). As in the sedentary group, we observed no sex (0.84±0.05 vs. 0.81±0.06, men and women, respectively, P=0.3), age (0.83±0.04 vs. 0.82±0.05, young and middle-aged adults, respectively, P>0.9) and weight status (0.81±0.06, 0.82±0.06, 0.82±0.05, for normal-weight, overweight and obese individuals, respectively, P>0.9) differences. The RER at Fat_{max} was between 0.67 and 0.7 in 5.7% (n=5) of participants, between 0.7 and 0.8 in 26.1% (n=23) of participants, and between a 0.8 and 0.9 in 68.2% (n=60) of participants. To note is that the graded exercise protocol total duration was 24.0±4.6 min when RER=1.0, while if we had considered that the graded exercise protocol ended at the highest registered RER at Fat_{max} (i.e. 0.9), the total duration would have been 15.6±5.8 min (mean difference 8.5±3.7 min).

DISCUSSION

Taken together, these findings suggest that a graded exercise protocol aiming to determine MFO and Fat_{max} could end when a RER=0.93 is reached in sedentary healthy adults, and when a RER=0.90 is reached in trained adults independently of sex, age, and weight status. Whereas these figures should be confirmed in other studies, we suggest reducing the RER from 1.0 to 0.95 to be sure that MFO is reached in outliers individuals.

More sophisticated data analysis approaches, such as 2nd or 3rd polynomial curve with intersection in (0,0) have been applied to accurately estimate MFO and Fat_{max} [18,19]. These methodologies require at least 4 fat oxidation values (preferably 6 or more) to determine MFO and Fat_{max}. Reducing the maximum RER from 1.0 to 0.95 could lead to fewer fat oxidation points, and may hamper the application of those methods. To this end, we used the baseline data of the above-mentioned cohorts to calculate MFO by a 3rd polynomial curve using all fat oxidation values when RER was ≤0.95 and when RER ≤1.0. No meaningful differences in MFO were observed between both methodologies (0.37±0.12 vs. 0.36±0.11 g/min, for RER≤1.0 and RER≤0.95 respectively; P=0.971). Similarly, there were no differences in MFO calculated with the measured-values data analysis approach (0.34±0.11 vs. 0.34±0.12 g/min, for RER≤1.0 and RER≤0.95 respectively; P=0.924). These findings suggest that reducing maximum RER to 0.95 does not affect the MFO estimation. Reducing maximum RER until 0.95 would allow to apply smaller workload increments without increasing the test duration, which would allow more fat oxidation values around Fat_{max}, increasing the accuracy of the MFO estimation.

Limitations

Our data should however be taken with caution since we conducted a treadmill test, and we do not know whether these findings can be extended to cycle ergometer test. Of note is also that our participants were healthy adults, thus future studies are needed to elucidate if these results can be applied to younger people or to patients. Moreover, future studies should confirm these findings in other populations of elite athletes or very well-trained individuals.

CONCLUSIONS

In summary, our results have important implications, and may allow to substantially reduce the graded exercise protocol duration to assess MFO and Fat_{max}. Further studies are needed to investigate the impact of reducing the RER criteria on the MFO and Fat_{max} accuracy, by means of increasing the stage duration to attain the steady-state and decreasing the workload increments magnitude.

REFERENCES

1 Goodpaster BH, Sparks LM. Metabolic Flexibility in Health and Disease. *Cell Metab* 2017; **25**: 1027–1036.

2 Galgani JE, Moro C, Ravussin E. Metabolic flexibility and insulin resistance. *AJP Endocrinol Metab* 2008; **295**: E1009–E1017.

3 Maunder E, Plews DJ, Kilding AE. Contextualising Maximal Fat Oxidation During Exercise: Determinants and Normative Values. *Front Physiol* 2018; **9**: 1–13.

4 Achten J, Gleeson M, Jeukendrup AE. Determination of the exercise intensity that elicits maximal fat oxidation. *Med Sci Sports Exerc* 2002; **34**: 92–97.

5 Amaro-Gahete FJ, Sanchez-Delgado G, Jurado-Fasoli L, De-la-O A, Castillo M, Helge JW *et al.* Assessment of maximal fat oxidation during exercise: a systematic review. *Scand J Med Sci Sport* 2019; : Under Revi.

6 Shephard RJ, Aoyagi Y. Measurement of human energy expenditure, with particular reference to field studies: An historical perspective. *Eur J Appl Physiol* 2012; **112**: 2785–2815.

7 Macfarlane DJ. Open-circuit respirometry: a historical review of portable gas analysis systems. *Eur J Appl Physiol* 2017; **117**: 2369–2386.

8 Bordenave S, Flavier S, Fedou C, Brun JF, Mercier J. Exercise calorimetry in sedentary patients: procedures based on short 3 min steps underestimate carbohydrate oxidation and overestimate lipid oxidation. *Diabetes Metab* 2007; **33**: 379–384.

9 Brun JF, Romain AJ, Mercier J. Lipoxmax ou oxydation maximale des lipides lors de l'exercice: Des mesures physiologiques aux applications cliniques. Réalités et incertitudes. *Sci Sport* 2011; **26**: 57–71.

10 Hultman E, Greenhaff PL. Skeletal muscle energy metabolism and fatigue during intense exercise in man. *Sci Prog* 1991; **75**: 361–70.

11 Sanchez-Delgado, G, Martinez-Tellez, B, Olza, J, Aguilera, CM, Labayen, I,... Ortega, FB, Ruiz J. Activating brown adipose tissue through exercise (ACTIBATE) in young adults: Rationale, design and methodology. *Contemp Clin Trials* 2015; **45**: 416–425.

12 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.

13 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; : 1–20.

14 Amaro-Gahete FJ, Sanchez-Delgado G, Ruiz JR. Commentary: Contextualising Maximal Fat Oxidation During Exercise:

Determinants and Normative Values. *Front Physiol* 2018; **9**: 1460.

15 Jeukendrup AE, Wallis GA. Measurement of substrate oxidation during exercise by means of gas exchange measurements. *Int J Sports Med* 2005; **26 Suppl 1**: S28-37.

16 Amaro-Gahete FJ, Sanchez-Delgado G, Alcantara JMA, Martinez-Tellez B, Acosta FM, Helge JW *et al.* Impact of data analysis methods for maximal fat oxidation estimation during exercise in sedentary adults. *Eur J Sport Sci* 2019; : 1–10.

17 Frayn KN. Calculation of substrate oxidation rates in vivo from gaseous exchange. *J Appl Physiol* 1983; **55**: 628–34.

18 Stisen AB, Stougaard O, Langfort J, Helge JW, Sahlin K, Madsen K. Maximal fat oxidation rates in endurance trained and untrained women. *Eur J Appl Physiol* 2006; **98**: 497–506.

19 Croci I, Borrani F, Byrne NM, Byrne N, Wood RE, Wood R *et al.* Reproducibility of Fatmax and fat oxidation rates during exercise in recreationally trained males. *PLoS One* 2014; **9**: e97930.

Diurnal variation of maximal fat oxidation rate in trained male athletes ***(Study 6)***

ABSTRACT

The purpose of this study was to analyze the diurnal variation of MFO and Fat$_{max}$ in trained male athletes.

A total of 12 endurance-trained male athletes aged 24.7±4.1 participated in the study. We measured MFO, Fat$_{max}$, VO_2max and VT2 with a graded exercise protocol performed on two days separated by one week. One test was performed in the morning and the other in the afternoon. We assessed the participants' chronotype using the HÖME questionnaire.

Results: Our results indicate that MFO and Fat$_{max}$ are greater in the afternoon than in the morning (Δ=13%, $P<0.001$ and Δ=6%, $P=0.001$, respectively), whereas there were similar VO_2max and VT2 in the morning than in the afternoon test (Δ=0.2%, $P=0.158$ and Δ=7%, $P=0.650$, respectively). There was a strong positive association between VO_2max and MFO in both morning and afternoon assessments ($R^2=0.783$; $P=0.001$ and $R^2=0.663$; $P<0.001$, respectively). Similarly, there was a positive association between VO_2max with Fat$_{max}$ in both morning and afternoon assessment ($R^2=0.406$; $P=0.024$ and $R^2=0.414$; $P=0.026$, respectively).

These findings suggest that the diurnal variation of MFO and Fat$_{max}$ may partially explain some of the observed diurnal variation in the performance of endurance sports.

BACKGROUND

Carbohydrates and fats are the primary substrates oxidized to fuel energy metabolism during exercise [1]. Humans predominantly store carbohydrates as glycogen in skeletal muscle and liver, and ~4g circulating in plasma as glucose [2]. However, these storage depots are limited, whereas human fat energy storage is effectively unlimited during prolonged exercise [3]. Therefore, the capacity to adapt fuel oxidation to fuel availability (known as metabolic flexibility) is a key determinant of endurance sport performance [4]. Therefore, MFO capacity during a graded exercise protocol is considered an important factor in endurance exercise performance as well and in cardiovascular health [5]. Moreover, another important variable is the exercise intensity at which MFO occurs, so called Fat_{max}. Both MFO and Fat_{max}, together with VO_2max, VO_2max percentage at VT2 and running economy are considered as important outcomes in endurance sports performance [5,6].

Endurance sport performance, specifically running and cycling performance, seems to present diurnal variation, being higher in the afternoon than in the morning [7]. This might be explained by a higher body temperature, higher neural activation and contractile properties of the skeletal muscle, or higher plasma catecholamine concentrations immediately after exercise in the afternoon than in the morning [8,9]. It has been observed a higher MFO and Fat_{max} in the afternoon than in the morning in non-athlete male students [10] and in untrained normal-weight and obese individuals [11]. Whether the observed diurnal variations in MFO and Fat_{max} also apply to endurance trained athletes is unknown. Moreover, despite it has been reported that individuals with higher VO_2max present greater muscle capacity to oxidize fat [12,13], the relation of VO_2max with MFO and Fat_{max} in endurance trained male athletes remains to be elucidated.

Therefore, the aims of this study were to analyze the diurnal variations of MFO and Fat_{max} in endurance trained male athletes. We also determined the diurnal variations of VO_2max and VT2, and examined the association of VO_2max and VT2 with MFO and Fat_{max}. We hypothesized that MFO, Fat_{max}, VO_2max and VT2 are higher in the afternoon compared with in the morning, and that VO_2max and VT2 are positively associated with MFO and Fat_{max} in endurance trained male athletes.

MATERIAL & METHODS

Participants

A total of 14 endurance trained male athletes aged 18-32 years voluntarily participated in the study. Two out of 14 participants did not meet the predetermined conditions (see below) for MFO and Fat_{max} measurements on one of the testing days and were retrospectively excluded from further statistical analyses. All athletes had extensive experience in endurance events and had a minimum of 2 years of cycling or running practice as a part of their main training schedule. They had a BMI between 18 and 25 kg/m^2, were nonsmokers, did not take any medication, and had no acute or chronic illness. All participants provided written informed consent to participate in the study, which was performed in accordance with the Declaration of Helsinki. Ethic approval was obtained from the University of Granada Research Ethics Committee (ethical approval code Nº 507/CEIH/2018).

Design and methodology

The study was conducted between March and April 2018. MFO and Fat_{max} were measured on 2 different days separated by 1 week. Measurements were performed between 8 am and 11 am (MFO-morning, Fat_{max}-morning, VO_2max-morning and VT2-morning), and between 5 and 8 pm in the afternoon (MFO-afternoon, Fat_{max}-afternoon, VO_2max-afternoon and VT2-afternoon). The test order (morning vs. afternoon) was randomized using a simple random function of the software MS Excel for Windows®.

Participants arrived at the laboratory by car or by bus (avoiding any physical activity) in a fasted state (between 7-10 hours). Participants were instructed to avoid moderate or vigorous physical activity 24 and 48 h before the testing day, respectively. A nutritionist prescribed an individualized pre-trial diet (i.e. 24hrs before each testing day: 2653±162 kcal; 50% carbohydrates, 30% fat and 20% protein) and the participants adhered to it of their own accord. When the tests were performed in the afternoon, we instructed to the participants to consume the same menu (same energy intake and % of macronutrient in each meal), at the same order than those consumed in the morning test (i.e. the breakfast in the morning test [24 hours ago] was the lunch in the afternoon test [24 hours ago]). Energy demand was determined using the Harris-Benedict equation based on body mass, height, and age. An activity factor of 1.8 was used [14].

On day 1, the weight and height were measured using a Seca scale and stadiometer (model 760, Electronic Column Scale, Hamburg, Germany), and the BMI was calculated as weight (kg)/height (m^2). Participants wore light clothing and no shoes during the measurements. FM was assessed by dual energy X-ray absorptiometry (Hologic Discovery Wii, Hologic, Bedford MA, USA). The participants also completed the HÖME questionnaire [15], which is a validated questionnaire that determines the participants' chronotype (morningness–eveningness). The questionnaire consists of 19 questions related to sleep/wake behaviour and yields scores ranging from 16 to 86. Based on the HÖME score, the participants were categorized into one of five chronotype categories: 16-30: definite evening type, 31-41: moderate evening type, 42-58: neither type, 59-69: moderate morning type and 70-86: definite morning type.

The resting metabolic rate was measured by IC during 15 minutes in peaceful and relaxing room (temperature: 22.6 ± 0.7ºC; humidity: 44.5 ± 6.1%). After that, a maximal walking speed protocol on a treadmill (H/P/cosmos pulsar, H/P/cosmos sports & medical GmbH, Nussdorf-Traunstein, Germany) was performed on the first day before the graded exercise protocol to determine MFO, Fat_{max}, VO_2max and VT2 adapted from a validated protocol [16]. In brief, the protocol started with a 3 minutes warm-up at 3.5 km/h, and 1 km/h speed increments were programmed every 3 minutes until the maximal walking speed was reached. Subsequently, the treadmill speed was constant, and the gradient was increased by 2% every 3 minutes until the RER was ≥ 1.0. Then, after a 5-minutes break, a maximal incremental exercise test, using the modified Balke protocol (3 min walking at 5.3 km/h and 1%, followed by increments of 1% every minute) until voluntary exhaustion was performed. The final 30 seconds of the VO_2 measurement was considered to be maximal (VO_2max) when the following conditions were met: (i) a plateau (an increase of <2ml/kg/min) in VO_2 with a further increasing workload; (ii) a heart rate at least higher than the age-predicted maximum minus 10 bpm; (iii) a respiratory-exchange ratio >1.1. If any of these criteria was not met, a VO_2peak value was taken, defined as the highest VO_2 measured over a 30 seconds period. VT2 was estimated from gas exchange data by two independent researchers following a validated standard methodology as previously described [17].

An oronasal mask (model 7400, Hans Rudolph Inc, Kansas City, MO, USA), equipped with a prevent™ metabolic flow sensor (Medgraphics Corp, Minnesota, USA) was fitted, and breath-by-breath respiratory measurements were recorded throughout the test with the use of an automated gas-analysis system (CPX Ultima CardiO2, Medical Graphics Corp, St Paul, USA). Gas analyzers were calibrated immediately before each graded exercise protocol according to the manufacturer's recommendations. Heart rate was recorded using a heart-rate monitor (Polar RS800, Polar Electro Inc., Woodbury, NY).

VO_2, VCO_2 and ventilation data were averaged over the most stable 5-consecutive-minute periods (after discarding the first 5 minutes) for analysis of the resting metabolic rate applying the Weir abbreviated equation (assuming negligible protein oxidation) and expressed as kcal/day: Resting metabolic rate = [3.9 (VO2) + 1.1 (VCO2)] * 1.44. On the other hand, VO_2, VCO_2 and ventilation data were averaged over and the last 60 s of each graded exercise protocol stage. Stoichiometric equations described by Frayn were used to calculate fat oxidation rates with the assumption that urinary nitrogen excretion was negligible [18] in all cases. Fat oxidation rates were plotted against the relative exercise intensity (% of VO_2max) and a third-degree polynomial regression was used to determine

MFO and Fat_{max} for each individual participant.

Statistical analysis

The determination of the sample size and power of the study are made based on the data of a pilot study. We considered MFO differences between morning and afternoon test in order to assess the sample size requirements for the one-way ANOVA. As a result, we expected to detect an effect size of 0.05 g/min considering a type I error of 0.05 with a statistical power of 0.85 with a minimum of 10 participants. Assuming a maximum loss of 20%, we decided to recruited a total of 12 participants.

Result are reported as the mean ± SD, otherwise stated. We used the Shapiro-Wilk test, visual check of histograms, and Q-Q plots to verify the normal distribution of all variables. A repeated-measures ANOVA was applied to determine differences between MFO-morning vs. MFO-afternoon, Fat_{max}-morning vs. Fat_{max}-afternoon, VO_2max-morning vs. VO_2max-afternoon and VT2-morning vs. VT2-afternoon. A one-way repeated-measures ANCOVA was conducted to study morning vs. afternoon differences including FM percentage, and chronotype as covariates.

To analyze the association of VO_2max and VT2 with MFO and Fat_{max} , we conducted a simple linear regression analysis as follows: (i) VO_2max-morning with MFO-morning, (ii) VO_2max-morning with Fat_{max}-morning, (iii) VT2-morning with MFO-morning, (iv) VT2-morning with Fat_{max}-morning, (v) VO_2max-afternoon with MFO-afternoon, (vi) VO_2max-afternoon with Fat_{max}-afternoon, (vii) VT2-afternoon with MFO-afternoon, and (viii) VT2-afternoon with Fat_{max}-afternoon. We also included FM percentage, and chronotype as covariates.

The analyses were conducted using the Statistical Package for Social Sciences (IBM Corporation, Chicago, IL, USA). For all statistical procedures, the significance level was set at $p \leq 0.05$.

RESULTS

Descriptive parameters of the study participants are listed in Table 1. Most of the participants did not fit in definite morning or definite evening chronotypes (~92%). The test order was morning-afternoon in 7 participants and afternoon-morning in 5 participants. Fasting time was similar in the morning and in the afternoon (8.4±1.2 vs. 8.2±1.0 hrs., respectively, P=0.554).

We observed significant differences between MFO-morning and MFO-afternoon (0.55±0.12 vs. 0.63±0.15 g/min, respectively; P<0.001, Figure 1A and 1B), which persisted after controlling for FM percentage and chronotype (P=0.023, and P<0.001, respectively).

Table 1. Descriptive parameters of study participants (n=12).

Age (years)	24.7	±	4.1
Weight (kg)	69.5	±	9.2
Height (m)	1.75	±	0.04
BMI (kg/m²)	22.7	±	2.3
FM (%)	16.7	±	3.7
Resting metabolic rate (kcal/day)	2096.8	±	212.8
Resting fat oxidation (g/min)	0.068	±	0.014
VO_2max (ml/kg/min)	63.8	±	9.6
HÖME questionnaire score	47.1	±	13.7
Definitive evening type (n [%])	1 [8.3]		
Moderate evening type (n [%])	2 [16.7]		
Neither type (n [%])	6 [50.0]		
Moderate morning type (n [%])	3 [25.0]		
Definite morning type (n [%])	0 [0]		

Values expressed as mean ± standard deviation.

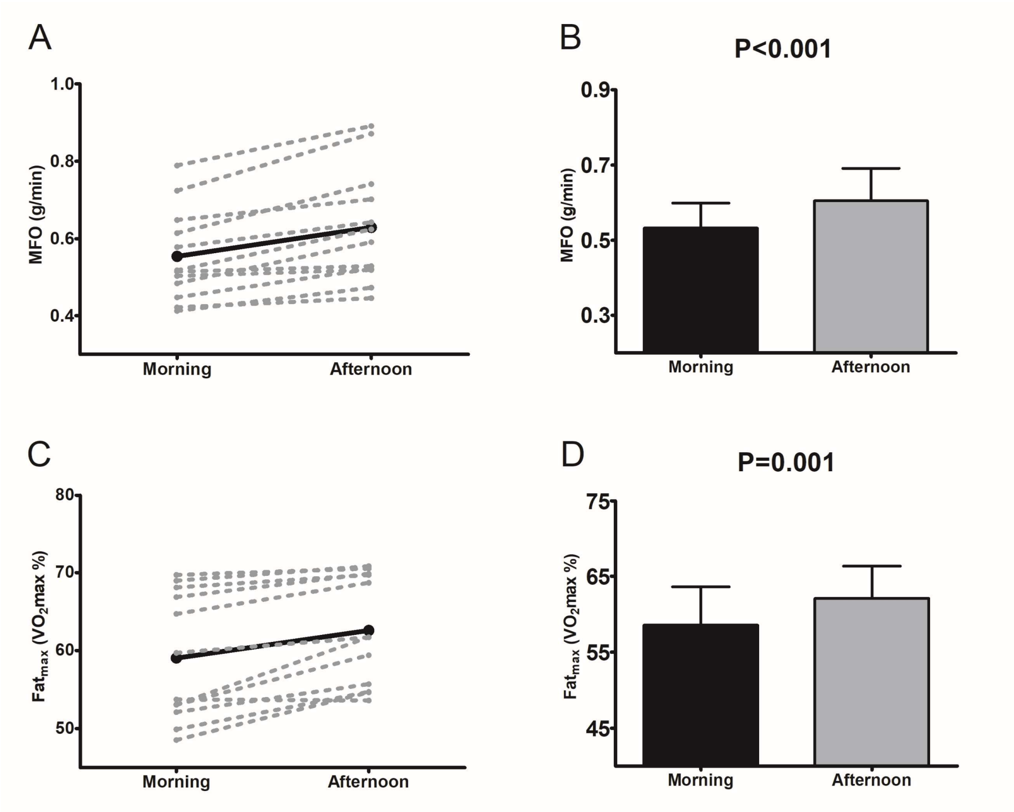

Figure 1. Maximal fat oxidation (MFO) in the morning (MFO-morning) and in the afternoon (MFO-afternoon) [Panel A and B], and the intensity which MFO occurs (Fatmax) in the morning (Fatmax-morning) and in the afternoon (Fatmax-afternoon) [Panel C and D]. Results are shown as the individual observations for each participant (gray lines), and as the mean for all participants (black line). P value obtained by repeated-measures ANOVA.

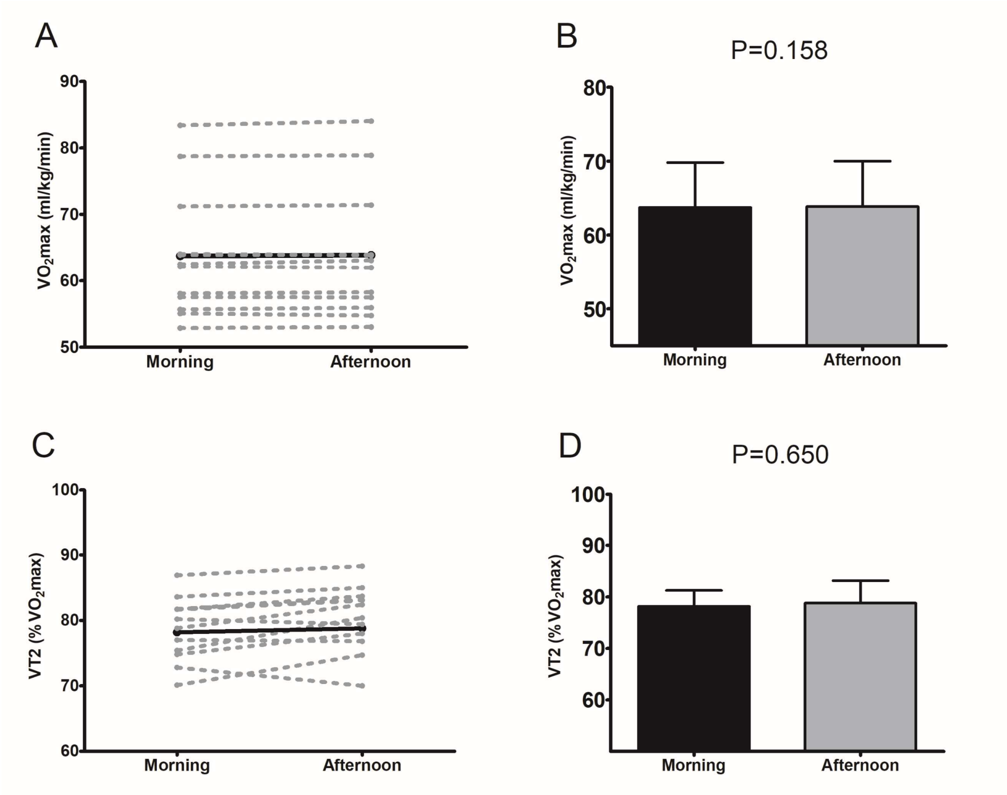

Figure 2. Maximal oxygen uptake (VO_2max) in the morning (VO2max-morning) and in the afternoon (VO2max -afternoon) [Panel A and B], and VO2max percentage in ventilatory threshold 2 (% of VO_2max VT2) in the morning (% of VO_2max VT2-morning) and in the afternoon (% of VO_2max -afternoon) [Panel C and D]. Results are shown as the individual observations for each participant (gray lines), and as the mean for all participants (black line). P value obtained by repeated-measures ANOVA.

Similarly, there were significant differences between Fat_{max}-morning and Fat_{max}-afternoon (59.0±8.1 vs. 62.6±7.0% of VO_2max, respectively; P=0.001, Figure 1C and 1D), that remained once FM percentage and chronotype were included in the model (P=0.018, and P<0.001, respectively).

There were no significant differences between VO_2max-morning and VO_2max-afternoon (63.7±9.5 vs. 63.9±9.7 ml/kg/min, respectively; P=0.158, Figure 2A and 2B), which persisted after controlling for FM percentage and chronotype (P=0.288, and P=0.561, respectively). Similarly, there were no significant differences between VT2-morning and VT2-afternoon (78.2±4.9 vs. 78.8±6.9% of VO_2max, respectively; P=0.650, Figure 2C and 2D), that remained once FM percentage and chronotype were included in the model (P=0.309, and P=0.784, respectively).

VO_2max was positively associated with MFO in both morning (P<0.001, Figure 3A) and afternoon assessments (P=0.001, Figure 3B).

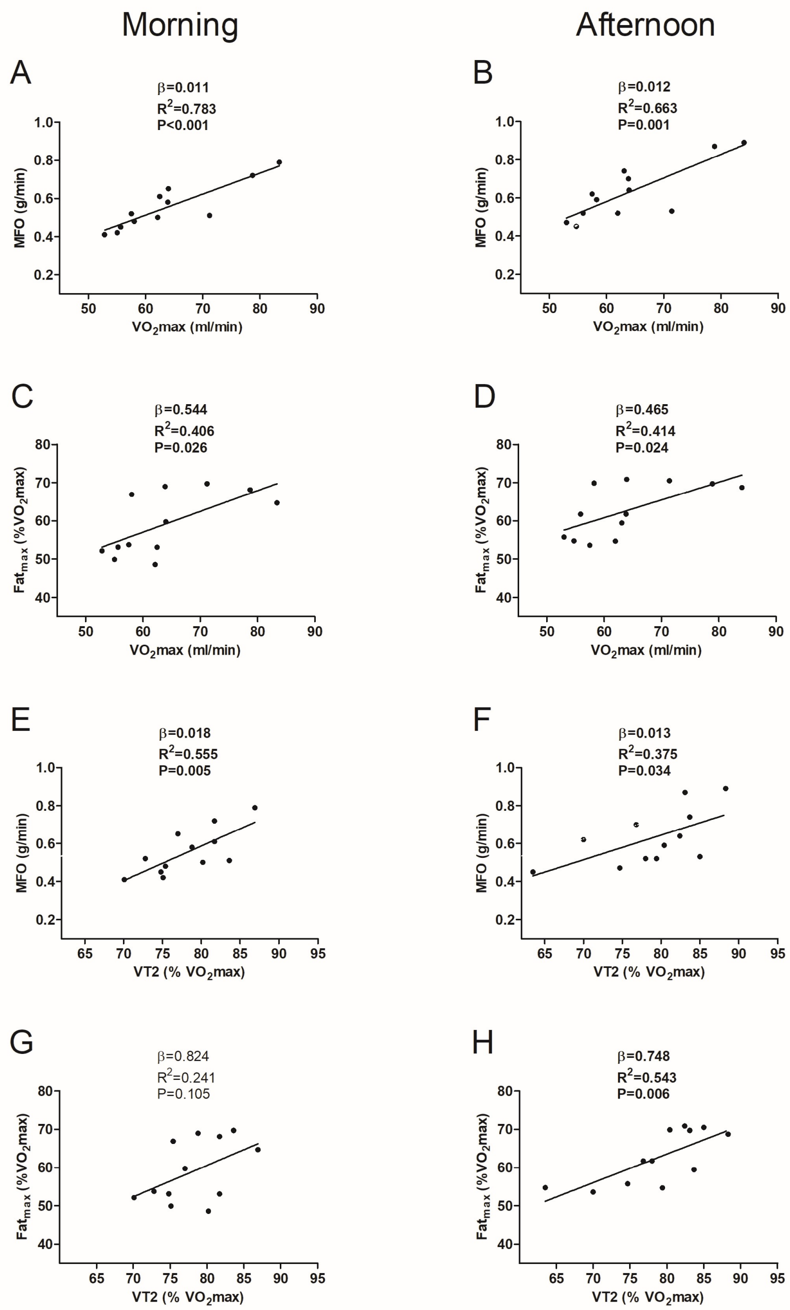

Figure 3. Association between (i) VO_2max-morning with MFO-morning (Figure 3A), (ii) VO_2max-afternoon with MFO-afternoon (Figure 3B), (iii) VO_2max-morning with Fat$_{max}$-morning (Figure 3C), (iv) VO_2max-afternoon with Fat$_{max}$-afternoon (Figure 3D), (v) VT2-morning with MFO-morning (Figure 3E), (vi) VT2-afternoon with MFO-afternoon (Figure 3F), (vii) VT2-morning with Fat$_{max}$-morning (Figure 3G), and (viii) VT2-afternoon with Fat$_{max}$-afternoon (Figure 3H). VO_2max (maximal oxygen uptake), VT2 (VO_2max percentage in ventilatory threshold 2), β (Unstandardized regression coefficient), R2 (coefficient of determination) and P value obtained from a simple linear regression analysis.

A positive association was observed between VO_2max and Fat_{max} in both morning (P=0.024, Figure 3C) and afternoon assessment (P=0.026, Figure 3D). VT2 was positively associated with MFO in both morning (P=0.005, Figure 3E) and afternoon assessments (P=0.034, Figure 3F).

A positive association was observed between VT2 and Fat_{max} in afternoon assessment (P=0.024, Figure 3H), while a tendency toward significance was noted in the morning assessment (P=0.105, Figure 3G). We repeated all the regression analysis after controlling for either FM or chronotype, and the results did not change (data not shown).

DISCUSSION

The main finding of this study shows that MFO and Fat_{max} are higher in the afternoon than in the morning in endurance trained male athletes. Moreover, we observed no differences in VO_2max and VT2 in the morning vs. afternoon. We also observed a significant positive association of VO_2max as well as VT2 with both MFO and Fat_{max}. These findings support the idea that the MFO and Fat_{max} diurnal variation should be considered for repeat laboratory testing in research, clinical, and athlete monitoring settings, since maintaining the same fasting time does not seem to nullify these effects. Additionally, these finding may partially explain the observed increased endurance sport performance in the afternoon, specifically in events limited by endogenous carbohydrate availability [7].

Our results extend those reported by others in untrained individuals [10,11]. Mohebbi et al. [11] reported that MFO and Fat_{max} were higher in the afternoon than in the morning in untrained normal-weight and obese individuals aged 19-25 years old. Similarly, Darvakh et al. [10] observed significantly greater MFO and Fat_{max} in the afternoon compared with in the morning in non-athlete male students. The MFO differences observed in the present study were larger than those observed by Mohebbi et al. [11] (14.5% vs. 8.9%) and Darvakh et al. [10] (14.5% vs. 6.7%), whereas Fat_{max} differences were smaller than those obtained by Mohebbi et al. [11] and Darvakh et al. [10] (6.1% vs. 12.2% and 10.7%, respectively) [10,11].

It is well-known that the catecholamine peak induced by exercise is higher in the afternoon than in the morning [9]. Considering that the catecholamine release activates the lipolysis in skeletal muscle and in adipose tissue [19], it seems reasonable that this will lead to increased plasma fatty acid content which could explain the elevated fat oxidation rates observed in the afternoon. However, a higher catecholamine release in the afternoon may also increase the glycogenolysis during exercise [20] producing a potential decrement of fat oxidation during exercise. Future studies are needed to investigate whether a higher plasma catecholamine concentration can induce higher MFO and Fat_{max} levels in the afternoon, since we have no data on exercise-induced catecholamine release. In addition, a number of studies suggest that body temperature, time to the exhaustion, and VO_2max in the afternoon are higher than in the morning in active and untrained individuals [11,21]. Moreover, our data indicate that both VO_2max and VT2 are similar in the morning and in the afternoon, which does not agree with others [11,21]. Other studies are warranted to determine diurnal differences in VO_2max and VT2 in trained athletes.

The association of VO_2max with MFO and Fat_{max} remains unclear, since controversial results have been reported. Several studies showed positive associations of VO_2max with MFO in moderately trained men (VO_2max ranged from 50-55ml/kg/min) [22], in trained males endurance athletes (VO_2max >70ml/kg/min) [23], in healthy young adults (VO_2max=43.9±7.2 ml/kg/min) [13], and in a very heterogenous group of 300 men and women (VO_2max=46.3±0.7 ml/kg/min) [12], which concur with the results obtained in our study. However, these findings differ from those obtained by others [24,25], who did not find significant associations between VO_2max and MFO in healthy trained individuals (VO_2max=58.0±1.6 ml/kg/min) [24], and in male ironman athletes (VO_2max ranged from 43.9–72.5 ml/kg/min) [25]. It has been suggested that this association is only present when heterogenous groups are compared [12,25,26]. These results concurred with our findings that showed a strong positive association between VO_2max and MFO in a heterogeneous cohort of endurance trained male athletes (VO_2max ranged from 52.9 to 83.7 ml/kg/min). The fact that individuals with higher VO_2max normally had greater capacity of the muscle to oxidize fat [27] could partially explain the observed association. In addition, it has previously reported that trained individuals use more fat at the same relative exercise intensity than untrained individuals in both longitudinal [28] and cross-sectional [26] training studies.

Previous studies suggested that greater endurance performance is most frequently observed in the afternoon [7]. Atkinson et al. [29] showed that aerobic cycling performance (measured by peak power) is greater in the afternoon than in the morning in trained cyclist. Moreover, Souissi et al. [21] also found greater peak power and VO_2max in the afternoon than in the morning, yet no differences were observed from morning to afternoon in VO_2max when corrected for total work done. Taken together, it is plausible that the diurnal variation of MFO and Fatmax might be the key factor in endurance performance diurnal variation, rather than of VO_2max and VT2, specifically in events limited by endogenous carbohydrate availability.

Limitations

The results of this study should be considered with caution. The lack of body temperature data and blood parameters assessments during the graded protocol test did not allow us to confirm whether metabolic and hormonal variables play a role in MFO and Fat_{max} diurnal variation. It should also be acknowledged that the present study was performed in endurance trained male athletes, thus these results cannot be extended to women or a sedentary population. Despite we established a fasted state (between 7-10 hours) as a pre-testing previous condition, a stricter control of fasting conditions should be considered in future studies (i.e. 8 hours), although our results remained after controlling by fasting time (data not shown). In addition, we do not know whether the differences found in MFO and Fat_{max} are determined by the individual chronotype, since the small sample size made it difficult to study. Finally, Croci et al. (2014) reported a CV ranging from 16 to 21% for MFO estimation determined from two progressive exercise protocols completed 3–7 days apart [30]. This variability may bias the results obtained by the current study.

CONCLUSIONS

In summary, our results indicate that MFO and Fat_{max} are greater in the afternoon than in the morning in endurance trained male athletes, whereas there is no diurnal variation in VO_2max and VT2. Moreover, we observed a positive strong association of VO_2max and VT2 with MFO and Fat_{max}. These data are relevant when scheduling training times, and specifically for coaches, who usually engage in athletic testing and monitor training session that can occur during different hours of the day, whenever the training intensity will be the Fat_{max}. Further studies are needed to investigate whether these results remain when a running or cycling protocol are used to estimate MFO and Fat_{max}.

REFERENCES

1 Jeukendrup AE. Regulation of fat metabolism in skeletal muscle. *Ann N Y Acad Sci* 2002; **967**: 217–235.

2 Jensen J, Rustad PI, Kolnes AJ, Lai Y-C. The Role of Skeletal Muscle Glycogen Breakdown for Regulation of Insulin Sensitivity by Exercise. *Front Physiol* 2011; **2**: 112.

3 Gonzalez JT, Fuchs CJ, Betts JA, van Loon LJC. Liver glycogen metabolism during and after prolonged endurance-type exercise. *Am J Physiol Metab* 2016; **311**: E543–E553.

4 Fernandez-Verdejo R, Bajpeyi S, Ravussin E, Galgani JE. Metabolic Flexibility to Lipid Availability During Exercise is Enhanced in Individuals with High Insulin Sensitivity. *Am J Physiol Endocrinol Metab* 2018.

5 Maunder E, Plews DJ, Kilding AE. Contextualising Maximal Fat Oxidation During Exercise: Determinants and Normative Values. *Front Physiol* 2018; **9**: 1–13.

6 Barnes KR, Kilding AE. Strategies to improve running economy. *Sport Med* 2015; **45**: 37–56.

7 Drust B, Waterhouse J, Atkinson G, Edwards B, Reilly T. Circadian Rhythms in Sports Performance—an Update. *Chronobiol Int* 2005; **22**: 21–44.

8 Teo W, Newton MJ, McGuigan MR. Circadian rhythms in exercise performance: Implications for hormonal and muscular adaptation. *J Sport Sci Med* 2011; **10**: 600–606.

9 Kim HK, Konishi M, Takahashi M, Tabata H, Endo N, Numao S *et al.* Effects of acute endurance exercise performed in the morning and evening on inflammatory cytokine and metabolic hormone responses. *PLoS One* 2015; **10**: 1–10.

10 Darvakh H, Nikbakht M. Effect of Circadian Rhythm on Peak of Maximal Fat Oxidation on Non-Athletic Men. *Zahedan J Res Med Sci* 2013; : 8–11.

11 Mohebbi H, Azizi M. Maximal fat oxidation at the different exercise intensity in obese and normal weight men in the morning and evening. *J Hum Sport Exerc* 2011; **6**: 49–58.

12 Venables MC, Achten J, Jeukendrup AE. Determinants of fat oxidation during exercise in healthy men and women: a cross-sectional study. *J Appl Physiol* 2005; **98**: 160–167.

13 Ponce González JG, Guadalupe-Grau A, Rodríguez-González FG, Torres-Peralta R, Morales-Alamo D, Rodríguez-García L *et al.* Androgen receptor gene polymorphisms and maximal fat oxidation in healthy men. A longitudinal study. *Nutr Hosp* 2017; **34**: 1089–1098.

14 Harris J, Benedict F. A biometric study of basal metabolism in man. *Proc Natl Acad Sci* 1918; **4**: 370–373.

15 Horne JA, Ostberg O. A self-assessment questionnaire to determine morningness-eveningness in human circadian rhythms. *Int J Chronobiol* 1976; **4**: 97–110.

16 Dandanell S, Præst CB, Søndergård SD, Skovborg C, Dela F, Larsen S *et al.* Determination of the exercise intensity that elicits maximal fat oxidation in individuals with obesity. *Appl Physiol Nutr Metab* 2017; **42**: 405–412.

17 Lucía A, Hoyos J, Chicharro JL. The slow component of VO2 in professional cyclists. *Br J Sports Med* 2000; **34**: 367–74.

18 Frayn KN. Calculation of substrate oxidation rates in vivo from gaseous exchange. *J Appl Physiol* 1983; **55**: 628–34.

19 Jocken JWE, Blaak EE. Catecholamine-induced lipolysis in adipose tissue and skeletal muscle in obesity. *Physiol Behav* 2008; **94**: 219–30.

20 Watt MJ, Howlett KF, Febbraio MA, Spriet LL, Hargreaves M. Adrenaline increases skeletal muscle glycogenolysis, pyruvate dehydrogenase activation and carbohydrate oxidation during moderate exercise in humans. *J Physiol* 2001; **534**: 269–78.

21 Souissi N, Bessot N, Chamari K, Gauthier A, Sesboüé B, Davenne D. Effect of time of day on aerobic contribution to the 30-s Wingate test performance. *Chronobiol Int* 2007; **24**: 739–748.

22 Croci I, Borrani F, Byrne NM, Wood RE, Hickman IJ, Cheneviere X *et al.* Reproducibility of Fatmax and fat oxidation rates during exercise in recreationally trained males. *PLoS One* 2014; **9**: e97930.

23 van Loon LJ, Greenhaff PL, Constantin-Teodosiu D, Saris WH, Wagenmakers AJ. The effects of increasing exercise intensity on muscle fuel utilisation in humans. *J Physiol* 2001; **536**: 295–304.

24 Astorino TA, Edmunds RM, Clark A, Gallant R, King L, Ordille GM *et al.* Change in maximal fat oxidation in response to different regimes of periodized high-intensity interval training (HIIT). *Eur J Appl Physiol* 2018; **117**: 745–755.

25 Frandsen J, Vest SD, Larsen S, Dela F, Helge JW. Maximal Fat Oxidation is Related to Performance in an Ironman Triathlon. *Int J Sports Med* 2017; **38**: 975–982.

26 Nordby P, Saltin B, Helge JW. Whole-body fat oxidation determined by graded exercise and indirect calorimetry: a role for muscle oxidative capacity? *Scand J Med Sci Sports* 2006; **16**: 209–214.

27 Venables MC, Achten J, Jeukendrup AE. Determinants of fat oxidation during exercise in healthy men and women: a cross-sectional study. *J Appl Physiol* 2005; **98**: 160–167.

28 Tan S, Wang J, Cao L, Guo Z, Wang Y. Positive effect of exercise training at maximal fat oxidation intensity on body composition and lipid metabolism in overweight middle-aged women. *Clin Physiol Funct Imaging* 2016; **36**: 225–230.

29 Atkinson G, Todd C, Reilly T, Waterhouse J. Diurnal variation in cycling performance: Influence of warm-up. *J Sports Sci* 2005; **23**: 321–329.

30 Croci I, Borrani F, Byrne NM, Byrne N,

Wood RE, Wood R *et al*. Reproducibility of Fatmax and fat oxidation rates during exercise in recreationally trained males. *PLoS One* 2014; **9**: e97930.

Normative values for maximal fat oxidation during exercise in sedentary adults ***(Study 7)***

ABSTRACT

Using a short-duration Graded exercise protocol and continuous IC, whole-body rates of fat and carbohydrate oxidation can be estimated across a range of exercise workloads, along with the individual MFO and Fat_{max}. These variables appear to have sport and health implications. After the discussion of the key determinants of MFO and Fat_{max} that should be considered during laboratory measurement, the present study provides MFO and Fat_{max} normative values collected in two different cohorts using a submaximal walking protocol. These normative values can be used to contextualize individual measurements and define research cohorts according their capacity for fat oxidation during exercise.

BACKGROUND

We read with interest the study by Maunder et al. where they elegantly synthesized the available evidence regarding the biological factors that affect MFO and Fat_{max} [1]. Moreover, they compiled data from previous studies and provided normative values for MFO and Fat_{max}. Although we appreciate the usefulness of this approach, there are several important aspects that need to be considered.

Firstly, as Maunder et al. [1] recognised, they provide percentiles for MFO and Fat_{max} derived from calculations based on mean and SD rather than in true percentiles. This approach assumes a normal distribution of data, which may not be the case in studies with relatively small sample size.

Secondly, due to the lack of definitions of physical activity or fitness level in overweight and obese populations, Maunder et al. [1] provided normative values for sedentary and physically active overweight/obese individuals without considering this important aspect. Several studies showed significant changes on MFO after an exercise intervention in overweight-obese individuals [2,3]. Therefore, the MFO and Fat_{max} normative values for the overweight and obese group should be considered with caution.

Thirdly, they compiled data from studies performed in cycloergometer. The mode of exercise (cycling, running, or walking) significantly influences MFO and Fat_{max} in young healthy and relatively fit individuals [4]. However, its influence on sedentary people is unknown. Thus, it remains to be elucidated whether the provided normative values for MFO and Fat_{max} apply to the treadmill test.

Finally, Maunder et al. [1] did not consider the potential effect of age on MFO and Fat_{max}, and, therefore, it was not taken into account in the normative values reported. Data from our laboratory (Table 1) suggest that age influences MFO, and, therefore, participants' age should be considered when providing normative values.

MATERIAL & METHODS

Here, we provide normative values by sex, weight status, and age for MFO and Fatmax (Table 1) of 167 (n=107 women) sedentary healthy individuals evaluated by a treadmill test. We determined the MFO and Fatmax in 125 young adults aged 22.1±2.2 years old [84 women, BMI: 25.0±4.8 kg/m^2] [5] and in 42 middle-aged adults aged 52.1±4.6 years old [23 women, BMI: 27.8±3.6 kg/m^2] [6] . We conducted a graded exercise protocol on a treadmill that started with a 3-minute warm-up at 3.5 km/h (gradient 0%) and continued with speed increments of 1 km/h every 3 minutes until the maximal walking speed was reached. The treadmill speed was kept constant with the gradient increasing by 2% every 3 minutes until the RER was ≥ 1.0 [7].

Table 1: Normative percentile values for maximal fat oxidation (MFO) and the exercise intensity at which maximal fat oxidation occurs (Fat^{max}) in sedentary individuals

	Population	N	MFO (g/min)			20th percentile	40th percentile	60th percentile	80th percentile	Fat_{max} ($\%VO_2max$)			20th percentile	40th percentile	60th percentile	80th percentile
ALL		167	0.34	±	0.10	0.24	0.30	0.35	0.42	44.2	±	12.4	33.2	39.6	44.6	54.1
BY SEX	Men	60	0.37	±	0.11	0.29	0.34	0.38	0.44	40.8	±	11.0	32.2	37.2	41.1	48.8
	Women	107	0.32	±	0.10	0.24	0.28	0.33	0.40	46.1	±	12.8	34.8	42.1	47.8	55.9
BY AGE	Young	125	0.36	±	0.11	0.28	0.32	0.36	0.44	44.0	±	13.3	32.5	39.0	43.2	54.6
	Middle-aged	42	0.29	±	0.08	0.22	0.24	0.28	0.38	44.7	±	9.5	35.8	41.4	46.0	53.4
BY SEX AND AGE	Young men	41	0.38	±	0.12	0.28	0.35	0.38	0.48	39.8	±	11.7	29.4	36.7	40.3	48.6
	Young women	84	0.35	±	0.09	0.28	0.31	0.35	0.42	46.1	±	13.6	34.4	41.6	46.6	60.4
	Middle-aged men	19	0.35	±	0.07	0.29	0.33	0.38	0.42	43.0	±	9.3	35.5	39.5	44.4	53.1
	Middle-aged women	23	0.24	±	0.03	0.22	0.23	0.24	0.27	46.1	±	9.6	39.0	42.7	50.1	54.4
BY BMI	Normal-weight	88	0.34	±	0.11	0.25	0.30	0.34	0.42	45.3	±	12.7	35.1	41.1	46.5	55.6
	Overweight	50	0.33	±	0.09	0.24	0.30	0.35	0.41	42.7	±	11.2	32.7	39.1	42.7	53.1
	Obese	29	0.36	±	0.12	0.26	0.30	0.39	0.45	43.3	±	13.5	32.3	39.1	42.0	54.8

Data are presented as mean (standard deviation). Abbreviations: BMI: Body mass index, min: Minute, VO_2max: maximal oxygen uptake.

Fat oxidation was calculated during the last 60 seconds of each step using a stoichiometric equation for respiratory gas exchange [8] disregarding protein oxidation. A third polynomial curve with intersection at 0;0 [9] was determined for each individual in order to determine MFO and Fat_{max}.

RESULTS AND DISCUSSION

Our results showed that absolute MFO was higher in men than in women (0.37±0.11 vs. 0.32±0.10 g/min, respectively, P=0.004, see Table 1), while Fat_{max} was lower in men than in women (40.8±10.99 vs. 46.1±12.84% of VO_2max, respectively, P=0.009, see Table 1). Considering the known sex-related differences in body composition, MFO relative to FFM might be more appropriate when conducting sex comparisons [1]. Our results showed that MFO relative to FFM (assessed by dual-energy X-ray absorptiometry) was lower in men than in women (0.050±0.026 vs. 0.084±0.043 g/min/kg, respectively, P<0.001). These findings concur with those presented by Maunder et al. [1], who showed that absolute MFO was greater in physically active men than in women (0.56 vs. 0.33 g/min, respectively), whereas Fat_{max} was slightly higher in physically active women than in men (56.0 vs. 51.0% of VO_2max, respectively).
A recent study described the MFO and Fat_{max} values in an athletic population across different ages, and showed large inter-individual differences regardless of the sport modality [10]. Our results showed significantly higher MFO in young compared with sedentary middle-aged adults (0.36±0.11 vs. 0.29±0.78 g/min, respectively, P<0.001), whereas no differences were observed in Fat_{max} (44.0±13.30 vs. 44.7±9.47% of VO_2max, respectively, P=0.753). Furthermore, we reported MFO and Fat_{max} normative values by weight status in sedentary adults. We observed similar MFO and Fat_{max} values in normal-weight, overweight, and obese individuals (MFO: 0.34±0.11, 0.33±0.09, and 0.36±0.12 g/min, respectively, P=0.494; Fat_{max}: 45.9±12.9 vs. 42.6±10.9 vs. 43.3±13.5% of VO_2max, respectively, P=0.146).
In contrast, Maunder et al. [1] showed lower MFO in obese individuals, which may be due to differences in training status, since Maunder et al. did not consider this dimension in the obese population.
It should be noted that the cohorts included in Maunder et al. review [1] performed a graded exercise protocol test after an overnight fast, whereas the participants in our study fasted only for 5-6 h. Previous studies suggested that the nutritional status plays an important role in MFO and Fat_{max} determination [1,11–13], and, therefore, fasting should be carefully considered when determining MFO and Fat_{max}.

CONCLUSIONS

We believe that the normative values provided by Maunder et al. [1] will be very useful when evaluating MFO and Fat_{max} both in research and in clinical settings. However, whenever possible, future studies should provide normative data by sex, age, training status, and weight status.

REFERENCES

1 Maunder E, Plews DJ, Kilding AE. Contextualising Maximal Fat Oxidation During Exercise: Determinants and Normative Values. *Front Physiol* 2018; **9**: 1–13.

2 Besnier F, Lenclume V, Gérardin P, Fianu A, Martinez J, Naty N *et al.* Individualized exercise training at maximal fat oxidation combined with fruit and vegetable-rich diet in overweight or obese women: The LIPOXmax-réunion randomized controlled trial. *PLoS One* 2015; **10**: 27–40.

3 Rosenkilde M, Reichkendler MH, Auerbach P, Bonne TC, Sjödin A, Ploug T *et al.* Changes in peak fat oxidation in response to different doses of endurance training. *Scand J Med Sci Sport* 2015; **25**: 41–52.

4 Mendelson M, Jinwala K, Wuyam B, Levy P, Flore P. Can crossover and maximal fat oxidation rate points be used equally for ergocycling and walking/running on a track? *Diabetes Metab* 2012; **38**: 264–270.

5 Sanchez-Delgado G, Martinez-Tellez B, Olza J, Aguilera CM, Labayen I, Ortega FB *et al.* Activating brown adipose tissue through exercise (ACTIBATE) in young adults: Rationale, design and methodology. *Contemp Clin Trials* 2015; **45**: 416–425.

6 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.

7 Jeukendrup AE, Wallis GA. Measurement of substrate oxidation during exercise by means of gas exchange measurements. *Int J Sports Med* 2005; **26 Suppl 1**: S28-37.

8 Frayn KN. Calculation of substrate oxidation rates in vivo from gaseous exchange. *J Appl Physiol* 1983; **55**: 628–34.

9 Croci I, Borrani F, Byrne NM, Byrne N, Wood RE, Wood R *et al.* Reproducibility of Fatmax and fat oxidation rates during exercise in recreationally trained males. *PLoS One* 2014; **9**: e97930.

10 Randell RK, Rollo I, Roberts TJ, Dalrymple KJ, Jeukendrup AE, Carter JM. Maximal Fat Oxidation Rates in an Athletic Population. *Med Sci Sports Exerc* 2017; **49**: 133–140.

11 Amaro-Gahete FJ, Ruiz JR. Methodological issues related to maximal fat oxidation rate during exercise : Comment on: Change in maximal fat oxidation in response to different regimes of periodized high-intensity interval training (HIIT). *Eur J Appl Physiol* 2018; **118**: 2029–2031.

12 Fletcher G, Eves FF, Glover EI, Robinson SL, Vernooij CA, Thompson JL *et al.* Dietary intake is independently associated with the maximal capacity for fat oxidation during exercise. *Am J Clin Nutr* 2017; **105**: 864–872.

13 Purdom T, Kravitz L, Dokladny K,

Mermier C. Understanding the factors that effect maximal fat oxidation. *J Int Soc Sports Nutr* 2018; **15**: 1–10.

Accuracy and validity of resting energy expenditure predictive equations in middle-aged adults ***(Study 8)***

ABSTRACT

IC is considered the reference method to determine REE, but its use in a clinical context is limited. Alternatively, there is a number REE predictive equations to estimate the REE. However, it has been shown that the available REE predictive equations could either overestimate or underestimate the REE as measured by IC. Moreover, the role of the weight status in the accuracy and validity of the REE predictive equations requires further attention. Therefore, this study aimed to determine the accuracy and validity of REE predictive equations in normal-weight, overweight and obese sedentary middle-aged adults.

A total of 73 sedentary middle-aged adults (53% women, 40-65 years old) participated in the study. We measured REE by indirect calorimetry strictly following the standard procedures and we compared it with 33 predictive equations.

The most accurate predictive equations in middle-aged sedentary adults were: (i) the equation of FAO/WHO/UNU in normal-weight individuals (50.0% of prediction accuracy) (ii) the equation of Livingston in overweight individuals (46.9% of prediction accuracy), and (iii) the equation of Owen in individuals with obesity (52.9% of prediction accuracy). Our study shows that the weight status plays an important role in the accuracy and validity of different REE predictive equations in middle-aged adults.

BACKGROUND

Obesity is associated with an increased morbidity and mortality risk, and is considered a significant burden to health care systems worldwide [1]. The amount of overweight and individuals with obesity has globally increased from 857 million to 2.1 billion during the last thirty years, becoming a public health problem of the current society [2]. Although the physiological mechanisms that determine or influence obesity are complex, several studies have shown that an energy imbalance between energy intake and energy expenditure is a predisposing factor for metabolic diseases [1].

Total energy expenditure is the sum of the REE, physical activity energy expenditure, and thermic effect of food. The REE accounts for more than 50% of the total daily energy expenditure [3]. IC is considered the reference method to determine the REE through the VO_2 and the VCO_2 [4]. However, the use of IC in a clinical context is limited due to its strict evaluation conditions, the high cost of the gas analyzer used for its measurement, and because these devices are not usually portable [5]. Alternatively, there is a number REE predictive equations to estimate the REE [6–24]. Previous studies have shown that the available REE predictive equations could either overestimate or underestimate the REE as measured by IC [25–28]. Furthermore, the majority of REE predictive equations were proposed decades ago, and they are based on some specific individual cohorts that have different biological and metabolic characteristics than the current population. Moreover, the role of the weight status in the accuracy and validity of the REE predictive equations requires further attention, since individuals with different weight status may have different amount of metabolically active tissues (FM vs. FFM), which could influence the REE estimation [29].

Therefore, the purpose of this study was to determine the accuracy and validity of REE predictive equations in normal-weight, overweight and obese sedentary middle-aged adults.

MATERIAL & METHODS

Participants

Seventy-three healthy sedentary adults (53% women), aged between 45 and 65, with a BMI range of 20-38 kg/m^2, Caucasian, non-physically active (<20 minutes on 3 days/week), and with stable weight (weight changes<5kg) over the last 6 months participated in the study. The participants were enrolled in the FIT-AGEING study (ClinicalTrials.gov: NCT03334357 [8-11-17]) [30]. We used the baseline data of the original study for data analysis. An informed consent was signed by each participant before they started the intervention program. The study was in accordance with the latest revision of the Helsinki Declaration, and it was approved by The Human Research Ethics Committee of the "*Junta de Andalucía*" [0838-N-2017].

Body composition

The weight was measured before the REE test using an electronic scale (Seca 760, Electronic Column Scale, Hamburg, Germany) to the nearest 0.1 kg. The height was also measured using a stadiometer (Seca 760, Electronic Column Scale, Hamburg, Germany) to the nearest 0.1 cm. The BMI was calculated as weight (kg)/height (m2) [31]. The body composition (FM, FFM, and LM) was determined by Dual Energy X-ray Absorptiometry (DXA, HOLOGIC, Discovery Wi).

Resting energy expenditure assessment by IC

The REE was evaluated by IC following the current recommendations to ensure the validity of the test [32,33]. The participants arrived at the laboratory at 8-9 a.m. after a 12-hour fasting period. The participants were asked not to perform any physical activity 48 hours before the test. The REE was evaluated in a quiet and relaxing room at a constant temperature (22.6±0.8ºC) and humidity (44.5±6.7%). The participants lay on a bed in a supine position, and they were asked not to fall asleep. The respiratory exchange was measured after resting for 30 minutes, using a CPX Ultima CardiO2 system (Medical Graphics Corp, St Paul, USA) and a neoprene facemask, equipped with a directconnect™ metabolic flow sensor (Medgraphics Corp, Minnesota, USA). The data were collected during 30 minutes. The first 5 minutes of each measurement were routinely discarded, and the most stable 5-minute steady state period was selected for the analysis (Breeze Software, MGC Diagnostic®, Breeze Suite 8.1.0.54 SP7) [34]. The steady-state criteria were established as: (i) <10% CV in VO_2, VCO_2, and ventilation, and (ii) <5% CV in RER [35,36]. The REE was calculated from the VO_2 consumed and the VCO_2 by using the Weir abbreviated equation assuming that urinary nitrogen excretion was negligible, and it was expressed as kcal/day [37]: REE = [3.9 (VO2) + 1.1 (VCO2)] x 1.44.

REE predictive equations

The National Library of Medicine's search service (PUBMED) was used to conduct a systematic search, combining the following keywords: 'Energy metabolism', 'Basal metabolism', 'Indirect calorimetry', and also additional terms (rest*, measure*, predict*', 'estimat*', 'equation*', and 'formula*').

We only selected the REE predictive equations that complied with the following criteria: (i) developed in adults, and (ii) based on weight, height, age, sex, and/or FM, FFM, and LM. We excluded the REE predictive equations: (i) conducted in patients with any disease or athlete cohorts, (ii) including a small sample size (n<50), or (iii) conducted in specific ethnic groups. A total of 33 predictive equations (see Table 1) were retained and used for analysis [6,7,16–24,8–15].

Statistical analysis

An ANCOVA was performed to compare measured (by IC) vs. predicted REE (by REE predictive equations) adjusting by age and sex. The BIAS (mean error between measured and predicted REE), the absolute differences (measured minus predicted REE in absolute terms), and the 95% limits of agreement were also analyzed. We determined the following two accuracy levels: (i) ±10% of measured REE, which included REE predicted values between 90% and 110% of the measured REE [38,39], considering underprediction when the estimation value was below 90%, and overprediction when the estimation value was above 110% of the measured REE, and (ii) ±5% of measured REE, which included REE predicted values between 95% and 105% of the measured REE, considering underprediction when the estimation value was below 95% and overprediction when the estimation value was above 105% of the measured REE. Repeated measures ANOVA across the REE predictive equations was used to determine differences between the REE predictive equation that presented the least absolute differences with measured REE, respectively. The heteroscedasticity was tested using the Bland-Altman method [40], which plots the difference between predicted and measured REE vs. the mean of predicted and measured REE. We conducted one-way ANOVA to determine differences across weight status categories (i.e. individuals with normal-weight, overweight and individuals with obesity) in the percentage of accurate prediction and mean differences between predicted and measured REE in absolute values of the most accurate predictive equations. We selected the most accurate REE predictive equations for each weight category based on the percentage of accurate prediction at ±10% of measured REE. If two or more REE predictive equations provide similar percentage of accurate prediction at ±10% of measured REE, we selected the most accurate REE predictive equation at ±5% of measured REE. The analyses were conducted using the SPSS version 25.0 (IBM Corporation, Chicago, IL, USA).

The analyses were conducted separately in normal-weight, overweight and obese. The results are expressed as mean ± SD, and the level of statistical significance was set at <0.05.

RESULTS

Table 2 shows the characteristics of the study sample.

Table 1: Resting energy expenditure predictive equations.

Reference	Participants	Statistics and cross-validation	REE predictive equations
Harris & Benedict (1919)	N=239 (136M; 103F), 21-70 y, 25-124.9 kg, 150-200 cm	M: r = 0.86, CL =211 F: r = 0.77, CL = 212	M: WT*13.7516+HTCM*5.0033–AGE*6.755+66.473 F: WT*9.5634+HTCM*1.8496-AGE*4.6756+655.0955
Roza et al. (1984)	N=337 (168M; 169F), 21-70 years, 25-124.9 kg, 150-200 cm	M: r = 0.86, CL = 213 F: r = 0.83, CL = 201	M: 13.397*WT+4.799*HTCM–5.677*AGE+88.362 F: 9.247*WT+3.098*HTCM–4.33*AGE+477.593
Bernstein et al. (1983)	N=202 (48 M; 154 F), 28-52 y, 60-204 kg, 157-182 cm, BMI>30	M: R^2=0.449 F: R^2=0.657 R^2=0.485	M: 11.02*WT+10.23*HTCM-5.8*AGE-1032 F: 7.48*WT-0.42*HTCM-3*AGE+844 19.02*FFM+3.72*FM-1.55*AGE+236.7
Owen et al. (1986)	N=104 (60 M; 44 F), 18–82 y, 60-171 kg (M) 43-153 kg (F), BMI 18–50	M: R^2=0.71 F: R^2=0.74 M: R^2=0.74 F: R^2=0.71	M: WT*10.2+879 F: WT*7.18+795 M: 22.3*FFM+290 F: 19.7*FFM+334
Mifflin et al. (1990)	N=498 (251 M; 248 F), N=264 normal weight (129 M; 135 F), N=234 individuals with obesity (122 M; 112 F), 19–78 y, BMI 17–42	R^2= 0.71 R^2= 0.64	9.99*WT+6.25*HTCM-4.92*AGE+166*SEX–161 19.7*FFM+413
Livingston et al. (2005)	N=655 (299 M; 356 F), 18–95 y, 33–278 kg	M: R^2= 0.77 F: R^2= 0.71	M: 293*$WT^{0.4330}$– 5.92*AGE F: 248*$WT^{0.4356}$–5.09*AGE

Schofield et al. (1985)	N=7,173, N=4,814>18 y, BMI 21–24 N= 3,388 Italians (47%), N=615 tropical residents, N= 322 Indian 114 published studies, N=7,173 subjects (11,000 values, includes group mean values); most European and North American subjects	M: 30-60y: r=0.60 >60y: r=0.74 F: 30-60y: r=0.68 >60y: r=0.73 M: 30-60y: r=0.60 >60y: r=0.74 F: 30-60y: r=0.68 >60y: r=0.73	M: 30-60y: 11,472*WT–873.1 >60y: 11.711*WT+587.7 F: 30-60y: 8,126*WT+845.6 >60y: 9.082*WT+658.5 M: 30-60y: 0.048*WT– 0.011*HTM+3.67 >60y: 0.038*WT+4.068*HTM+3.491 F: 30-60y: 0.034*WT+0.006*HTM+3.53 >60y: 0.033*WT+1.917*HTM+0.074
FAO (1985)	Equation based on Schofield et al (1985); database extended to 11,000 subjects	M: 30-60y; r=0.6 >60y: r=0.79 F: 30-60y: r= 0.7 >60y: R=0.74 M: 30-60y: r=0.6 >60y: 0.84 F: 30-60y: r=0.7 >60y: r=0.82	M: 30-60y: 11.6*WT+879 >60y: 13.5*WT+487 F: 30-60y: 8.7*WT+829 >60y: 10.5*WT+596 M: 30-60y: 11.3*Weight–16*Height+901 >60y: 8.8*WT+1128*HTM–1071 F: 30-60y: 8.7*WT–25*HTM+865 >60y: 9.2*WT+637*HTM–302
Henry et al. (2005)	N=10,552 (5794 M; 4702 F)	M: 30-60y: r=0.742 >60y: r=0.776 F: 30-60y: r=0.690 >60y: 0.786 M: 30-60y: r=0.756 >60y: r=0.789 F: 30-60y: r=0.713 >60y: 0.805	M: 30-60y: 0.0592*WT+2.48 >60y: 0.0563*WT+2.15 F: 30-60y: 0.0407*WT+2.9 >60y: 0.0424*WT+2.38 M: 30-60y: 0.0476*WT+2.26*HTM–0.574 >60y: 0.0478*WT+2.26*HTM–1.07 F: 30-60y: 0.0342*WT+2.1*HTM–0.0486 >60y: 0.0356*WT+1.76*HTM+0.0448

Muller et al. (2004)	N=2,528 (1027 M; 1501 F), 5–80 y, BMI >25	r=0.83	0.047*WT– 0.01452*AGE+1.009*SEX+3.21
		r=0.79	BMI 25–30: 0.04507*WT-0.01553*AGE+1.006*SEX+3.407
		r=0.84	BMI >30: 0.05*WT-0.01586*AGE+1.103*SEX+2.924
		r=0.83	0.05192*FFM+0.04036*FM+0.869*SEX-0.01181*AGE+2.992
		r=0.79	BMI 25–30: 0.03776*FFM+0.03013*FM+0.93*SEX-0.01196*AGE+3.928
		r=0.84	BMI >30: 0.05685*FFM+0.04022*FM+0.808*SEX-0.01402*AGE+2.818
Korth et al. (2007)	N=104 (50 M; 54 F), 21–68 y, BMI 18-41	r = 0.84, R^2= 0.71, SE = 788	41.5*WT+35.0*HTCM+1107.4*SEX-19.1*AGE-1731.2
		r = 0.86, R^2= 0.74, SE = 732	108.1*FFM+1231
De Lorenzo et al. (2001)	N=320 (127 M; 193 F), 18–59 y, BMI 17–40	F: R^2=0.597, SE=650	M: 53.284*WT+20.957*HTCM–23.859*AGE+487
		M: R^2=0.597, SE=581	F: 46.322*WT+15.744*HTCM–16.66*AGE+944
Lazzer et al. (2007)	N= 346 (164 M; 182 F), 20–65 y, mean BMI 45 (50% FM)	M: R^2=0.68, SE=1.14	M: 0.048*WT+4.655*HTM-0.020*AGE-3.605
		F: R^2=0.66, SE=0.56	F: 0.042*WT+3.619*HTM-2.678
Johnstone et al. (2006)	N=150 (43 M; 107 F), 21–64 y, BMI 17–49	R^2=0.774	90.2*FFM+31.6*FM-12.2*AGE+1613
Weijs et al. (2010)	N=536 F, >19 y, BMI >28	R^2 = 0.69, SEE = 204	WT*14.038+HTCM*4.498+SEX*137.566−AGE*0.977−221.631

Frankenfield (2015)	N=337, >18 y	R^2=0.84	BMI≥30: WT*10−AGE*5+SEX*274+865 BMI<30: WT*11-AGE*6+SEX*230+838 BMI≥30: WT*10+HTCM*3−AGE*5+SEX*244+440 BMI<30: WT*10+HTCM*3−AGE*5+SEX*207+454
De la Cruz et al. (2014)	N=134 (67 M; 67 F), 19-65 y	R^2=0.68	1376,4–308*SEX***+11,1*WT–8*AGE
Willis et al. (2015)	N=159, 18-30 y, BMI mean 30.7	R^2=0.77	11.2*WT−7.2*AGE+237.6*SEX+780.3
Cunningham (1980)	N=223 (120M; 103F)	R^2=0.7	500 + 22*Lean Body Mass
Huang (2004)	N=1088 (279M; 759F)	R^2=0.737 R^2=0.723	10.158*WT+3.933*HTCM–1.44*Age+273.821*Sex+60.655 14.118*FFM+9.367*FM–1.515*Age + 220.863*Sex+521.995
De Luis et al. (2006)	N=200 (60 M; 140 F), >20 y, BMI>30	M: R^2=0.70 F: R^2=0.70	M: 58.6+(6.1*WT)+(1023.7*HTM)–(9.5*AGE) F: 1272.5+(9.8*WT)–(61.6*HTM)–(8.2*AGE)

Abbreviations: M, male; F, female; y, years of age; kg, kilograms; cm, centimetres; BMI, body mass index; WT, weight; HTCM, height in centimetres; FFM, fat free mass; FM, fat mass; HTM, height in meters; r and r^2 values of the correlation between each predictive equation and the indirect calorimetry measurement in the original paper; CL, confident limit. ***Female*1, male*0.

Table 2. Descriptive parameters

	All (n=73)		Normal-weight (n=24)		Overweight (n=32)		Individuals with obesity (n=17)	
	Men (n=35)	Women (n=38)	Men (n=9)	Women (n=15)	Men (n=13)	Women (n=19)	Men (n=13)	Women (n=4)
Age (years)	54.4±5.3	52.9±5.1	55±5.4	53.1±4.6	54.4±5.8	53.1±5.3	53.9±4.9	51.8±6.9
Weight (kg)	86.36±11.05	66.36±10.04	73.03±5.68	58.94±5.55	86.19±5.98	68.40±6.61	95.76±7.73	84.46±9.91
Height (m)	175.8±6.5	160.9±6.0	178.2±5.0	162.2±4.9	177.7±6.6	159.8±6.6	172.3±6.2	161.6±7.3
Fat mass (%)	34.59±7.89	45.51±7.51	28.35±5.53	40.02±4.46	33.03±6.14	49.34±7.62	40.46±7.00	47.91±2.03
Fat free mass (kg)	56.04±6.88	36.01±6.54	52.28±5.36	35.32±4.02	57.77±6.8	34.85±7.20	56.92±7.35	44.11±6.47
Lean mass (kg)	53.41±6.71	34.08±6.37	49.70±5.15	33.42±3.97	55.13±6.60	32.94±7.03	54.27±7.22	41.97±6.08
REE (Kcal/day)	1796±196	1291±175	1763±130	1238±190	1806±258	1291±140	1808±173	1495±151

Data are expressed as mean ± standard deviation. Abbreviations: REE, resting energy expenditure.

In normal-weight individuals (see Figure 1A and Table 3), the Schofield [21] and FAO/WHO/UNU [22] predictive equations presented 66.7% of prediction accuracy, 20.8% underpredictions, and 12.5% overpredictions (accurate prediction ±10%). Nevertheless, when a severe accurate estimation (±5%) was applied, the equation of FAO/WHO/UNU [22] provided 50.0% of prediction accuracy and the equation of Schofield [21] 45.8% of prediction accuracy (mean absolute differences: 131 ± 138 and 129 ± 132 Kcal/day, respectively). Repeated measures ANOVA showed significant differences (all P<0.001) when comparing the REE estimation by the equation of FAO/WHO/UNU [22] vs. the equations of Owen [17,18] and Mifflin [19] (see Figure 1B). The results persisted including age and sex as a covariate (all P>0.3).

Figures 2A and 2B show the percentage of prediction accuracy in all REE predictive equations and mean absolute values differences between predicted and measured REE in overweight participants, respectively. The equations of Livingston [20] and Huang [15] provided a similar percentage of prediction accuracy (75%) when ±10% of accurate estimation was applied. However, when a severe accurate estimation filter (±5%) was applied, the equation of Livingston [20] showed the highest percentage of prediction accuracy (46.9% vs. 43.8%, respectively). The absolute differences were 117 ± 122 and 114 ± 109 Kcal/day for Livingston´s [20] and Huang´s REE predictive equations [15], respectively (see Table 4). An interaction effect in ANCOVA analysis was observed adjusting by age in the equations of Schofield [21] (P=0.003) and Owen [17,18] (P=0.042), whereas no sex interaction was observed in the model (P>0.4).

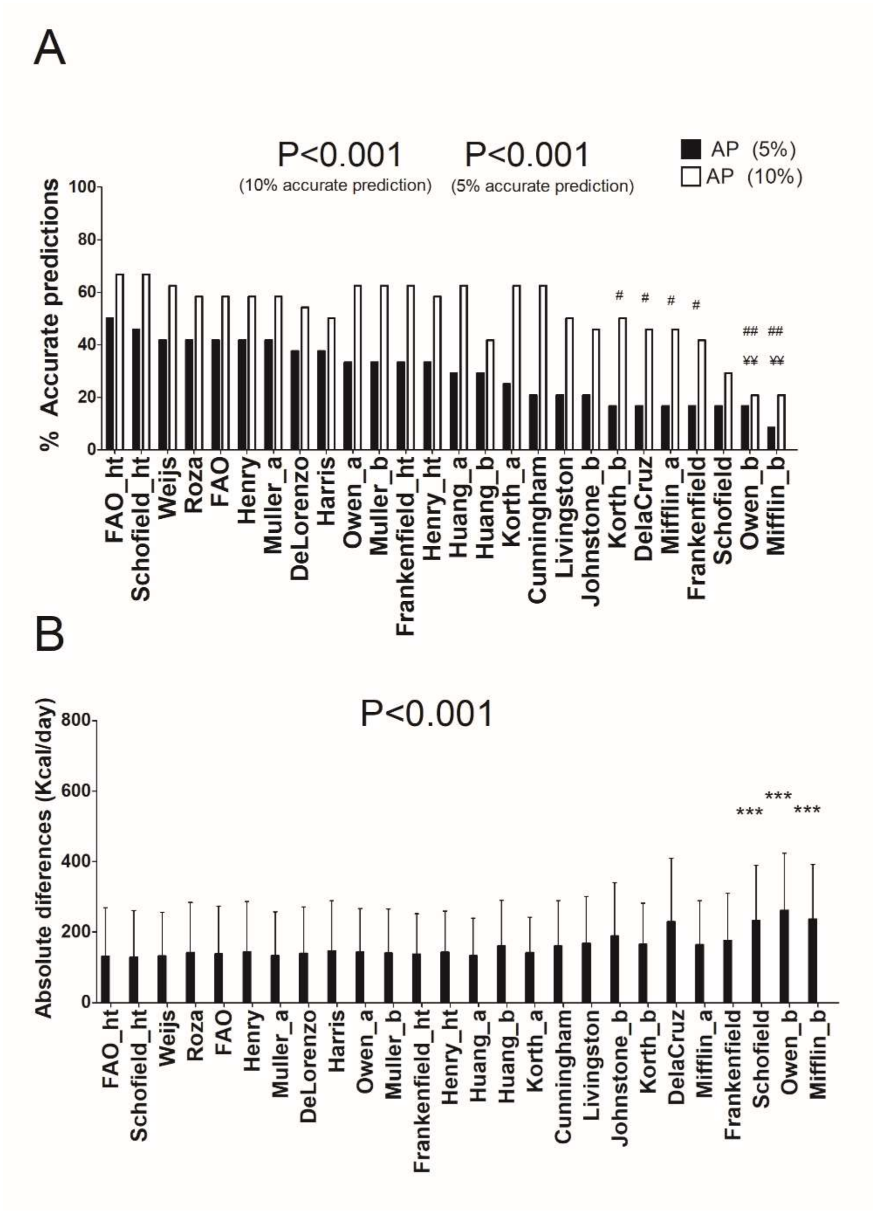

Figure 1. Percentage of accurate prediction of resting energy predictive equations and mean differences between predicted and measured resting energy expenditure in absolute values in normal-weight individuals. A: Percentage of prediction accuracy at 5% and 10% of resting energy expenditure. B: Mean (SD) differences between predicted and measured resting energy expenditure in absolute values. P value of repeated measures ANOVA (with Bonferroni post-hoc analysis) among the predictive equations. * = P<0.05; ** = P<0.01; *** = P<0.001 when compared with the predictive equation that presented the least absolute differences with resting energy expenditure measured (FAO_ht). ¥ = P<0.05; ¥ ¥ = P<0.01; ¥ ¥ ¥ = P<0.001 when compared with the predictive equation that presented the best resting energy expenditure prediction accuracy (10%) with resting energy expenditure measured (FAO_ht). # = P<0.05; ## = P<0.01; ### = P<0.001 when compared with the predictive equation that presented the best resting energy expenditure prediction accuracy (5%) with resting energy expenditure measured (FAO_ht). AP: Accurate prediction. "_a" refers to predictive equations which require only anthropometric parameters to calculate REE, "_b" refers to predictive equations which require body composition parameters to calculate REE, and "_ht" refers to predictive equations which are proposed by the same author and include height.

Table 3: Validity of resting energy expenditure (REE) predictive equations in normal-weight adults.

REE predictive equation	**N**	**[1]REE (Kcal/day)**	**P value ANCOVA[2]**	**Mean BIAS[3] (Kcal/day)**	**Lower limit of agreement (Kcal/day)**	**Higher limit of agreement (Kcal/day)**	**Mean absolute differences[4] (Kcal/day)**	**Percentage of accurate predictions (10%)[5]**	**Percentage of under predictions (10%)[6]**	**Percentage of over predictions (10%)[7]**	**Percentage of accurate predictions (5%)[8]**	**Percentage of under predictions (5%)[9]**	**Percentage of over predictions (5%)[10]**
Harris & Benedict	24	1391 ± 174	0.660	45	-405	586	146 ± 143	50.0	20.8	29.2	37.5	20.8	41.7
Roza	24	1413 ± 171	0.668	23	-424	565	142 ± 143	58.3	20.8	20.8	41.7	25.0	33.3
Owen_a	24	1370 ± 206	0.764	65	-376	508	143 ± 124	62.5	12.5	25.0	33.3	20.8	45.8
Owen_b	24	1192 ± 230	0.486	244	-166	671	262 ± 162	20.8	4.2	75.0	16.7	4.2	79.2
Mifflin_a	24	1329 ± 210	0.951	106	-287	566	164 ± 126	45.8	8.3	45.8	16.7	20.8	62.5
Mifflin_b	24	1234 ± 187	0.728	201	-242	671	237 ± 156	20.8	8.3	70.8	8.3	12.5	79.2
Livingston	24	1327 ± 188	0.725	108	-332	582	168 ± 133	50.0	8.3	41.7	20.8	16.7	62.5
Schofield	24	1217 ± 337	0.521	219	-125	548	232 ± 158	29.2	4.2	66.7	16.7	4.2	79.2
Schofield_ht	24	1453 ± 172	0.745	-17	-484	363	129 ± 132	66.7	20.8	12.5	45.8	25.0	29.2
FAO	24	1460 ± 167	0.458	-24	-499	347	138 ± 136	58.3	20.8	20.8	41.7	29.2	29.2
FAO_ht	24	1460 ± 164	0.785	-24	-496	373	131 ± 138	66.7	20.8	12.5	50.0	25.0	25.0
Henry	24	1433 ± 160	0.818	2	-455	551	143 ± 144	58.3	20.8	20.8	41.7	25.0	33.3
Henry_ht	24	1508 ± 287	0.463	-72	-431	237	143 ± 117	58.3	33.3	8.3	33.3	45.8	20.8
Muller_a	24	1392 ± 204	0.846	44	-382	498	133 ± 125	58.3	16.7	25.0	41.7	20.8	37.5
Muller_b	24	1375 ± 200	0.918	61	-368	517	140 ± 126	62.5	12.5	25.0	33.3	20.8	45.8
Korth_a	24	1483 ± 274	0.989	-47	-379	339	141 ± 101	62.5	29.2	8.3	25.0	45.8	29.2
Korth_b	24	1370 ± 245	0.743	65	-321	500	165 ± 117	50.0	12.5	37.5	16.7	29.2	54.2

De Lorenzo	24	1407 ± 189	0.738	29	-393	543	139 ± 133	54.2	20.8	25.0	37.5	25.0	37.5
Johnstone_b	24	1297 ± 193	0.973	139	-284	656	189 ± 152	45.8	8.3	45.8	20.8	12.5	66.7
Weijs	24	1435 ± 220	0.855	0	-382	502	132 ± 124	62.5	20.8	16.7	41.7	29.2	29.2
Frankenfield	24	1308 ± 196	0.725	128	-306	586	176 ± 134	41.7	8.3	50.0	16.7	12.5	70.8
Frankenfield_ht	24	1379 ± 225	0.855	57	-341	479	137 ± 115	62.5	8.3	29.2	33.3	20.8	45.8
De la Cruz	24	1603 ± 240	0.076	-167	-748	186	229 ± 181	45.8	45.8	8.3	16.7	66.7	16.7
Cunningham	24	1370 ± 201	0.899	66	-357	524	160 ± 129	62.5	12.5	25.0	20.8	20.8	58.3
Huang_a	24	1400 ± 245	0.717	36	-341	446	133 ± 107	62.5	12.5	25.0	29.2	25.0	45.8
Huang_b	24	1323 ± 223	0.761	113	-295	550	161 ± 129	41.7	8.3	50.0	29.2	12.5	58.3

[1]REE obtained by predictive equations (Mean±SD); [2]P value of the main effect of ANCOVA comparing measured and predicted REE adjusting for age; [3]Mean error between measured value and predictive equation (measured – predicted); [4]Mean of absolute differences between measured and predictive value (Mean±SD); [5]Percentage of subjects predicted by this predictive equation within ±10% of the measured value; [6]Percentage of subjects predicted by this predictive equation <10% of the measured value; [7]Percentage of subjects predicted by this predictive equation >10% of the measured value; [8]Percentage of subjects predicted by this predictive equation within ±10% of the measured value; [9]Percentage of subjects predicted by this predictive equation <10% of the measured value; [10]Percentage of subjects predicted by this predictive equation >10% of the measured value. *P<0.05, **P<0.01, ***P<0.001, ANCOVA test. "_a" refers to predictive equations which required only anthropometric parameters to calculate REE, "_b" refers to predictive equations which required body composition parameters to calculate REE, and "_ht" refers to predictive equations which are proposed by the same author and include height.

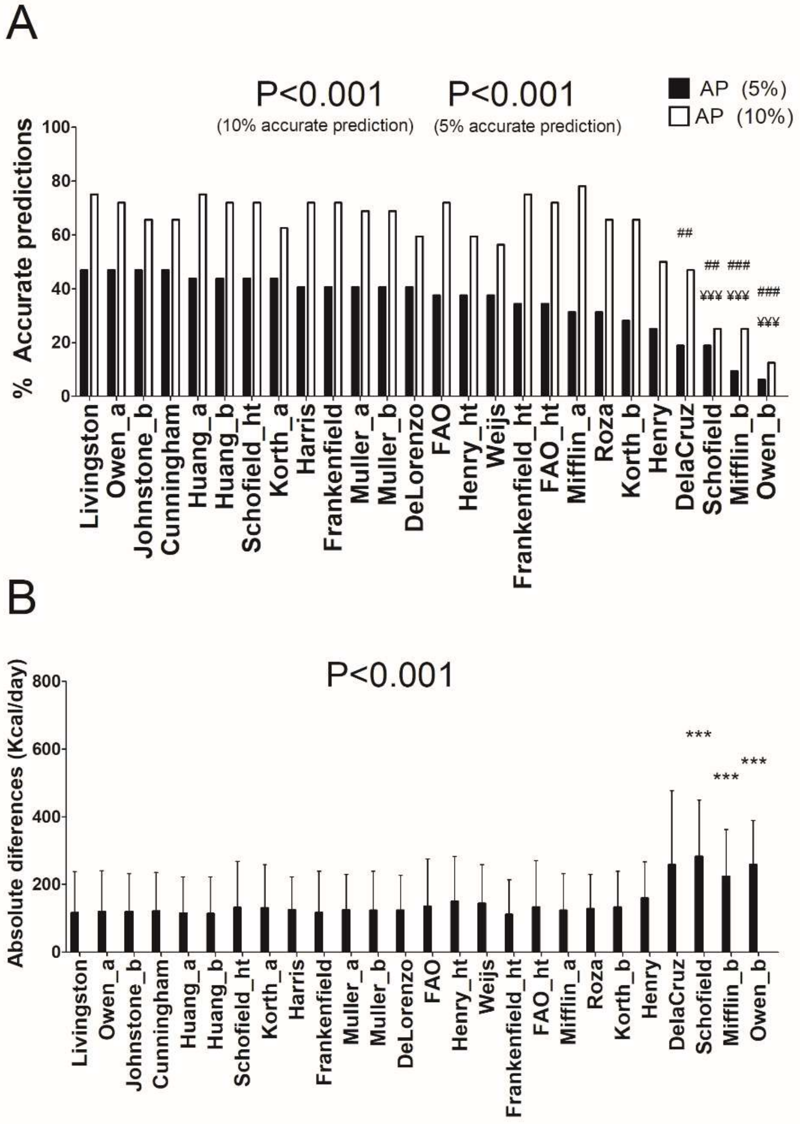

Figure 2. Percentage of accurate prediction of resting energy predictive equations and mean differences between predicted and measured resting energy expenditure in absolute values in overweight individuals. A: Percentage of prediction accuracy at 5% and 10% of resting energy expenditure. B: Mean (SD) differences between predicted and measured resting energy expenditure in absolute values. P value of repeated measures ANOVA (with Bonferroni post-hoc analysis) among the predictive equations. * = P<0.05; ** = P<0.01; *** = P<0.001 when compared with the predictive equation that presented the least absolute differences with resting energy expenditure measured (Livingston). ¥ = P<0.05; ¥¥ = P<0.01; ¥¥¥ = P<0.001 when compared with the predictive equation that presented the best resting energy expenditure prediction accuracy (10%) with resting energy expenditure measured (Roza). # = P<0.05; ## = P<0.01; ### = P<0.001 when compared with the predictive equation that presented the best resting energy expenditure prediction accuracy (5%) with resting energy expenditure measured (Livingston). AP: Accurate prediction. "_a" refers to predictive equations which require only anthropometric parameters to calculate REE, "_b" refers to predictive equations which require body composition parameters to calculate REE, and "_ht" refers to predictive equations which are proposed by the same author and include height.

Table 4: Validity of resting energy expenditure (REE) predictive equations in overweight adults.

REE predictive equation	N	REE[1] (Kcal/day)	P value ANCOVA[2]	Mean BIAS[3] (Kcal/day)	Lower limit of agreement (Kcal/day)	Higher limit of agreement (Kcal/day)	Mean absolute differences[4] (Kcal/day)	Percentage of accurate predictions (10%)[5]	Percentage of under predictions (10%)[6]	Percentage of over predictions (10%)[7]	Percentage of accurate predictions (5%)[8]	Percentage of under predictions (5%)[9]	Percentage of over predictions (5%)[10]
Harris & Benedict	32	1526 ± 231	0,285	-25	-340	456	125 ± 98	71.9	18.8	9.4	40.6	40.6	18.8
Roza	32	1543 ± 229	0,282	-42	-362	447	128 ± 102	65.6	25.0	9.4	31.3	53.1	15.6
Owen_a	32	1478 ± 241	0,008	23	-360	516	120 ± 121	71.9	12.5	15.6	46.9	21.9	31.3
Owen_b	32	1249 ± 312	0,042	251	-117	549	259 ± 131	12.5	0.0	87.5	6.3	3.1	90.6
Mifflin_a	32	1442 ± 250	0,189	58	-299	538	123 ± 110	78.1	3.1	18.8	31.3	18.8	50.0
Mifflin_b	32	1283 ± 263	0,071	216	-97	598	224 ± 139	25.0	0.0	75.0	9.4	3.1	87.5
Livingston	32	1455 ± 216	0,142	46	-249	562	117 ± 122	75.0	9.4	15.6	46.9	18.8	34.4
Schofield	32	1241 ± 336	0,003	260	-147	606	282 ± 167	25.0	0.0	75.0	18.8	6.3	75.0
Schofield_ht	32	1444 ± 194	0,228	57	-222	607	131 ± 137	71.9	6.3	21.9	43.8	18.8	37.5
FAO	32	1449 ± 188	0,544	51	-232	590	135 ± 140	71.9	6.3	21.9	37.5	31.3	31.3
FAO_ht	32	1451 ± 185	0,193	49	-249	618	133 ± 139	71.9	6.3	21.9	34.4	31.3	34.4
Henry	32	1586 ± 198	0,282	-86	-351	447	160 ± 107	50.0	40.6	9.4	25.0	62.5	12.5
Henry_ht	32	1615 ± 339	0,892	-115	-474	219	150 ± 132	59.4	37.5	3.1	37.5	56.3	6.3
Muller_a	32	1527 ± 226	0,117	-26	-351	481	124 ± 105	68.8	18.8	12.5	40.6	43.8	15.6
Muller_b	32	1500 ± 204	0,092	1	-305	532	123 ± 116	68.8	15.6	15.6	40.6	34.4	25.0
Korth_a	32	1596 ± 316	0,336	-95	-545	300	130 ± 129	62.5	34.4	3.1	43.8	50.0	6.3
Korth_b	32	1434 ± 345	0,423	66	-313	422	132 ± 107	65.6	3.1	31.3	28.1	18.8	53.1
De Lorenzo	32	1543 ± 236	0,319	-42	-369	444	124 ± 104	59.4	31.3	9.4	40.6	43.8	15.6

Johnstone_b	32	1418 ± 267	0,36	83	-222	473	120 ± 112	65.6	3.1	31.3	46.9	9.4	43.8
Weijs	32	1595 ± 260	0,158	-95	-484	374	144 ± 115	56.3	37.5	6.3	37.5	53.1	9.4
Frankenfield	32	1442 ± 225	0,921	59	-242	564	118 ± 122	71.9	6.3	21.9	40.6	18.8	40.6
Frankenfield_ht	32	1498 ± 257	0,823	2	-358	470	111 ± 103	75.0	12.5	12.5	34.4	37.5	28.1
De la Cruz	32	1520 ± 359	0,599	-19	-600	754	258 ± 219	46.9	34.4	18.8	18.8	53.1	28.1
Cunningham	32	1423 ± 285	0,731	78	-259	417	121 ± 114	65.6	3.1	31.3	46.9	9.4	43.8
Huang_a	32	1520 ± 276	0,529	-20	-438	430	115 ± 108	75.0	15.6	9.4	43.8	31.3	25.0
Huang_b	32	1449 ± 262	0,464	52	-317	491	114 ± 109	71.9	3.1	25.0	43.8	15.6	40.6

[1]REE obtained by predictive equations (Mean±SD); [2]P value of the main effect of ANCOVA comparing measured and predicted REE adjusting for age; [3]Mean error between measured value and predictive equation (measured – predicted); [4]Mean of absolute differences between measured and predictive value (Mean±SD); [5]Percentage of subjects predicted by this predictive equation within ±10% of the measured value; [6]Percentage of subjects predicted by this predictive equation <10% of the measured value; [7]Percentage of subjects predicted by this predictive equation >10% of the measured value; [8]Percentage of subjects predicted by this predictive equation within ±10% of the measured value; [9]Percentage of subjects predicted by this predictive equation <10% of the measured value; [10]Percentage of subjects predicted by this predictive equation >10% of the measured value. *P<0.05, **P<0.01, ***P<0.001, ANCOVA test. "_a" refers to predictive equations which required only anthropometric parameters to calculate REE, "_b" refers to predictive equations which required body composition parameters to calculate REE, and "_ht" refers to predictive equations which are proposed by the same author and include height.

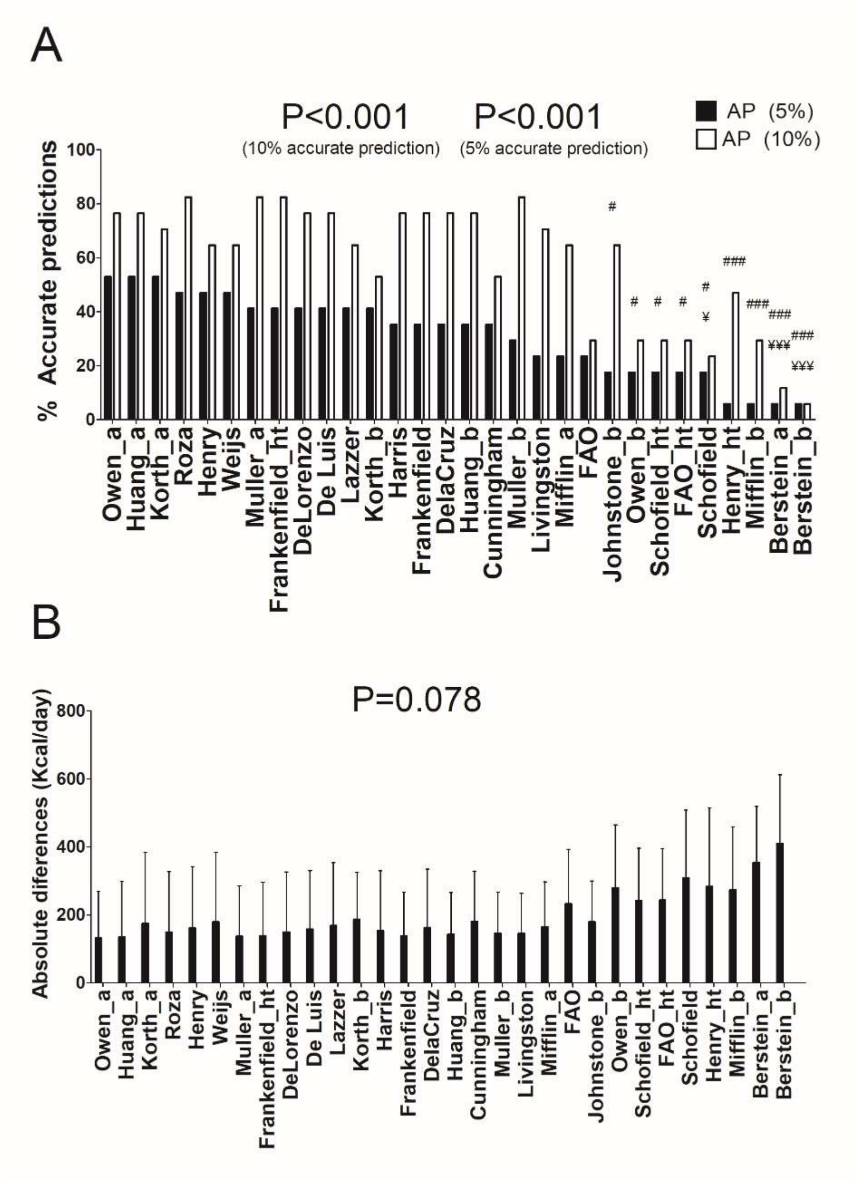

Figure 3. Percentage of accurate prediction of resting energy predictive equations and mean differences between predicted and measured resting energy expenditure in absolute values in individuals with obesity. A: Percentage of prediction accuracy at 5% and 10% of resting energy expenditure. B: Mean (SD) differences between predicted and measured resting energy expenditure in absolute values. P value of repeated measures ANOVA (with Bonferroni post-hoc analysis) among the predictive equations. * = P<0.05; ** = P<0.01; *** = P<0.001 when compared with the predictive equation that presented the least absolute differences with resting energy expenditure measured (Owen_a). ¥ = P<0.05; ¥ ¥ = P<0.01; ¥ ¥ ¥ = P<0.001 when compared with the predictive equation that presented the best resting energy expenditure prediction accuracy (10%) with resting energy expenditure measured (Roza). # = P<0.05; ## = P<0.01; ### = P<0.001 when compared with the predictive equation that presented the best resting energy expenditure prediction accuracy (5%) with resting energy expenditure measured (Owen_a). AP: Accurate prediction. "_a" refers to predictive equations which require only anthropometric parameters to calculate REE, "_b" refers to predictive equations which require body composition parameters to calculate REE, and "_ht" refers to predictive equations which are proposed by the same author and include height.

Table 5: Validity of resting energy expenditure (REE) predictive equations in individuals with obesity.

REE predictive equation	N	REE[1] (Kcal/day)	P value ANCOVA[2]	Mean BIAS[3] (Kcal/day)	Lower limit of agreement (Kcal/day)	Higher limit of agreement (Kcal/day)	Mean absolute differences[4] (Kcal/day)	Percentage of accurate predictions (10%)[5]	Percentage of under predictions (10%)[6]	Percentage of over predictions (10%)[7]	Percentage of accurate predictions (5%)[8]	Percentage of under predictions (5%)[9]	Percentage of over predictions (5%)[10]
Harris & Benedict	17	1796 ± 216	0.059	-61	-748	177	153 ± 177	76.5	17.6	5.9	35.3	35.3	29.4
Roza	17	1808 ± 211	0.062	-73	-747	151	149 ± 179	82.4	17.6	0.0	47.1	41.2	11.8
Bernstein_a	17	1422 ± 175	0.196	313	-346	594	354 ± 166	11.8	5.9	82.4	5.9	5.9	88.2
Bernstein_b	17	1325 ± 165	0.574	410	-4	694	410 ± 202	5.9	0.0	94.1	5.9	0.0	94.1
Owen_a	17	1749 ± 212	0.513	-14	-591	245	132 ± 138	76.5	11.8	11.8	52.9	23.5	23.5
Owen_b	17	1477 ± 217	0.688	258	-111	598	280 ± 185	29.4	0.0	70.6	17.6	5.9	76.5
Mifflin_a	17	1695 ± 198	0.076	40	-587	271	164 ± 133	64.7	5.9	29.4	23.5	17.6	58.8
Mifflin_b	17	1475 ± 176	0.613	260	-89	593	273 ± 187	29.4	0.0	70.6	5.9	5.9	88.2
Livingston	17	1710 ± 178	0.154	25	-535	247	145 ± 119	70.6	5.9	23.5	23.5	23.5	52.9
Schofield	17	1462 ± 281	0.983	273	-305	669	309 ± 200	23.5	5.9	70.6	17.6	5.9	76.5
Schofield_ht	17	1528 ± 182	0.17	206	-304	504	242 ± 154	29.4	5.9	64.7	17.6	5.9	76.5
FAO	17	1541 ± 186	0.137	194	-321	501	232 ± 162	29.4	5.9	64.7	23.5	5.9	70.6
FAO_ht	17	1526 ± 171	0.18	209	-292	503	243 ± 152	29.4	5.9	64.7	17.6	5.9	76.5
Henry	17	1866 ± 186	0.05	-131	-743	97	161 ± 181	64.7	35.3	0.0	47.1	47.1	5.9

Henry_ht	17	1953 ± 339	0.053	-218	-963	210	283 ± 232	47.1	47.1	5.9	5.9	70.6	23.5
Muller_a	17	1791 ± 194	0.121	-56	-655	164	137 ± 149	82.4	17.6	0.0	41.2	35.3	23.5
Muller_b	17	1750 ± 185	0.143	-16	-566	217	145 ± 123	82.4	11.8	5.9	29.4	29.4	41.2
Korth_a	17	1888 ± 246	0.06	-153	-826	59	174 ± 210	70.6	29.4	0.0	52.9	47.1	0.0
Korth_b	17	1686 ± 230	0.34	49	-311	435	187 ± 139	52.9	11.8	35.3	41.2	23.5	35.3
De Lorenzo	17	1813 ± 203	0.047	-78	-737	153	149 ± 177	76.5	23.5	0.0	41.2	41.2	17.6
Lazzer	17	1827 ± 205	0.038	-92	-779	178	168 ± 186	64.7	29.4	5.9	41.2	41.2	17.6
Johnstone_b	17	1687 ± 186	0.112	48	-438	327	179 ± 120	64.7	11.8	23.5	17.6	29.4	52.9
Weijs	17	1902 ± 205	0.055	-167	-826	63	178 ± 206	64.7	35.3	0.0	47.1	52.9	0.0
Frankenfield	17	1738 ± 192	0.921	-4	-579	213	138 ± 129	76.5	5.9	17.6	35.3	23.5	41.2
Frankenfield_ht	17	1800 ± 202	0.823	-65	-671	153	139 ± 157	82.4	17.6	0.0	41.2	35.3	23.5
De la Cruz	17	1721 ± 222	0.599	14	-633	535	162 ± 173	76.5	5.9	17.6	35.3	29.4	35.3
Cunningham	17	1630 ± 190	0.731	104	-239	460	181 ± 148	52.9	11.8	35.3	35.3	17.6	47.1
Huang_a	17	1807 ± 216	0.529	-72	-686	137	135 ± 165	76.5	23.5	0.0	52.9	29.4	17.6
Huang_b	17	1738 ± 196	0.464	-3	-552	222	143 ± 123	76.5	11.8	11.8	35.3	23.5	41.2
De Luis	17	1820 ± 195	0.333	-85	-705	150	158 ± 173	76.5	23.5	0.0	41.2	41.2	17.6

[1]REE obtained by predictive equations (Mean±SD); [2]P value of the main effect of ANCOVA comparing measured and predicted REE adjusting for age; [3]Mean error between measured value and predictive equation (measured – predicted); [4]Mean of absolute differences between measured and predictive value (Mean±SD); [5]Percentage of subjects predicted by this predictive equation within ±10% of the measured value; [6]Percentage of subjects predicted by this predictive equation <10% of the measured value; [7]Percentage of subjects predicted by this predictive equation >10% of the measured value; [8]Percentage of subjects predicted by this predictive equation within ±10% of the measured value; [9]Percentage of subjects predicted by this predictive equation <10% of the measured value; [10]Percentage of subjects predicted by this predictive equation >10% of the measured value. *P<0.05, **P<0.01, ***P<0.001, ANCOVA test. "_a" refers to predictive equations which required only anthropometric parameters to calculate REE, "_b" refers to predictive equations which required body composition parameters to calculate REE, and "_ht" refers to predictive equations which are proposed by the same author and include height.

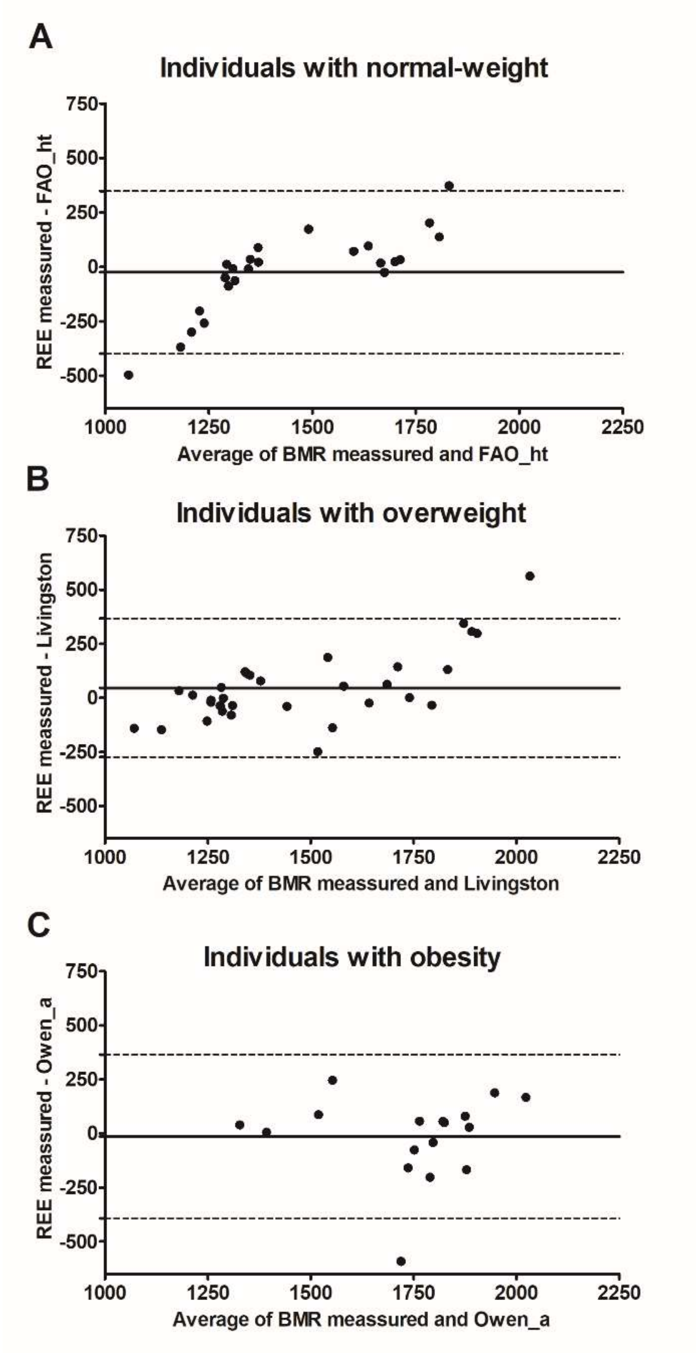

Figure 4. Bland-Altman plots for selected resting energy expenditure (REE) predictive equations. The solid lines represent the mean difference (BIAS) between predicted and measured REE. The upper and lower dashed lines represent the 95% limits of agreement. REE: Resting Energy Expenditure. "_a" refers to predictive equations which required only anthropometric parameters to calculate REE and "_ht" refers to predictive equations which are proposed by the same author and include height.

Figures 2A and 2B show the percentage of prediction accuracy in all REE predictive equations and mean absolute values differences between predicted and measured REE in overweight participants, respectively. The equations of Livingston [20] and Huang [15] provided a similar percentage of prediction accuracy (75%) when ±10% of accurate estimation was applied. However, when a severe accurate estimation filter (±5%) was applied, the equation of Livingston [20] showed the highest percentage of prediction accuracy (46.9% vs. 43.8%, respectively). The absolute differences were 117 ± 122 and 114 ± 109 Kcal/day for Livingston´s [20] and Huang´s REE predictive equations [15], respectively (see Table 4). An interaction effect in ANCOVA analysis was observed adjusting by age in the equations of Schofield [21] (P=0.003) and Owen [17,18] (P=0.042), whereas no sex interaction was observed in the model (P>0.4). We also noted significant differences (all P<0.01) when we compared the REE estimation (in absolute values) by the equation of Livingston [20] vs. the equations of Schofield [21], Mifflin [19] and Owen [17,18] (see Figure 2B).

In individuals with obesity, several REE predictive equations provided 82.4% of prediction accuracy (accurate prediction ±10%, see Figure 3A) [7,24,37], yet when a severe accurate estimation was applied (±5% of measured REE), the equation of Owen [17,18] showed the highest accuracy (52.9% of prediction accuracy; absolute differences: 132 ± 138 Kcal/day). An interaction effect was observed adjusting by age in De Lorenzo [41] and Lazzer [42] predictive equations (both P<0.05, see Table 5), whereas no interaction was observed adding sex in the model (P>0.2). Repeated measures ANOVA did not show significant differences between all predictive equations in terms of absolute differences (P=0.078) (see Figure 3B).

Figure 4 shows Bland-Altman plots for the 3 selected REE predictive equations and measured REE by weight status. The limits of agreement were the following: (i) -496 to 373 Kcal/day in normal-weight participants (using the equation of FAO/WHO/UNU [22], see Figure 4A and Table 3), (ii) -249 to 562 Kcal/day in overweight participants (using the equation of Livingston [20], see Figure 4B and Table 4), and (iii) -591 to 245 Kcal/day in individuals with obesity (using the equation of Owen [17,18], see Figure 4C and Table 5).

Figure 5 shows the comparison of the most accurate predictive equations for individuals with normal-weight, individuals with overweight and individuals with obesity, respectively, by weight status. We observed significant differences in percentage of accurate predictions applying both ±10% and ±5% of measured REE criteria in FAO_ht, Livingston and Owen_a predictive equations (All P<0.001, see Figure 5A).

No significant differences were noted comparing mean differences between predicted and measured REE in absolute values by weight status in Livingston and Owen_a predictive equations (All P>0.313, see Figure 5B), while significant differences were observed considering FAO_ht equation (P=0.023, see Figure 5B).

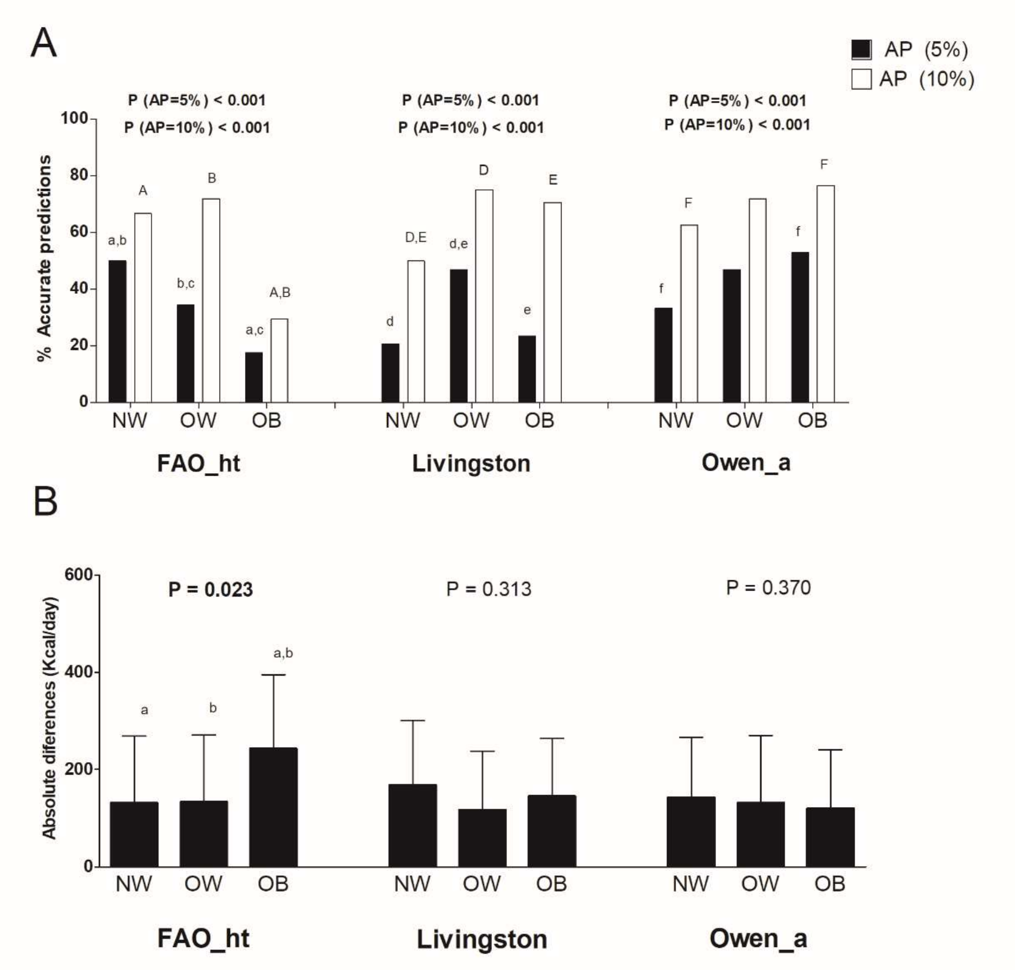

Figure 5. Percentage of accurate prediction of the most accurate predictive equations and mean differences between predicted and measured resting energy expenditure in absolute values by weight status. A: Percentage of prediction accuracy at 5% and 10% of resting energy expenditure. B: Mean (SD) differences between predicted and measured resting energy expenditure in absolute values. P value of an ANOVA (with Bonferroni post-hoc analysis) across weight status. Similar letters (i.e. a-a, b-b) indicate significant differences (P<0.05) considering Bonferroni post-hoc analysis. AP: Accurate prediction. "_a" refers to predictive equations which require only anthropometric parameters to calculate REE, and "_ht" refers to predictive equations which are proposed by the same author and include height.

DISCUSSION

The present study shows the most accurate predictive equations by weight status in sedentary middle-aged adults: (i) the equation of FAO/WHO/UNU [22] in normal-weight individuals, (ii) the equation of Livingston [20] in overweight individuals, and (iii) the equation of Owen [17,18] in individuals with obesity. Moreover, there were significant differences in percentage of accurate prediction when comparing the REE estimated values provided by the most accurate predictive equations for each weight status category. We also provide a flowchart decision tree to choose the REE predictive equation by weight status (see Figure 6) considering (i) the % of prediction accuracy applying an accuracy level of ±5%, and (ii) the % of prediction accuracy applying an accuracy level of ±10%.

Our results suggest that the best equation to estimate REE in normal-weight adults is the equation of FAO/WHO/UNU [22]. Our results differ from another study [37] that showed that the equation of Mifflin [19] was the most accurate REE predictive equation (68% of accuracy prediction) when an accuracy level of ±5% was applied. These differences could be explained due to the lack of details reported by Frankenfield et al. [37] regarding the IC analysis criteria to determine the REE measurement and the inclusion of a heterogeneous individual population. In a cohort of Belgian normal-weight women, the most accurate REE predictive equation was the Huang equation [43], with 71% of prediction accuracy. Our results also revealed a good prediction accuracy with the equation of Huang (62.5% of prediction accuracy) [43]. However, these differences might be explained by three specific facts: (i) Weijs et al. [43] only considered women, (ii) they selected 20-minute steady state periods to obtain the REE measurement (we selected the most stable 5-minute steady state period), and (iii) the gas analyzer device was different in both studies.

Our results provide more evidence for the use of the Livingston equation [20] in overweight individuals (46.9% prediction accuracy, mean absolute differences: 116.5 ± 121.9 Kcal/day), and concur with another study performed in normal-weight, overweight and individuals with obesity (55% prediction accuracy) [44]. However, a systematic review conducted in overweight individuals [45] reported higher accuracy when the Harris-Benedict predictive equation [6] was applied (62.7% of prediction accuracy), whereas we obtained 49.9% of prediction accuracy when using the same equation. These facts could be explained by the inclusion of numerous studies with different gas collection systems (e.g. direct calorimetry vs. IC), different gas analyzers used to determine REE (e.g. Vmax Encore n29, Viasys Healthcare vs. Oxycon Pro, Erich Jaeger GmbH, Hoechberg, Germany, between others), and also different population groups (e.g. overweight U.S. adults vs. normal-weight European women).

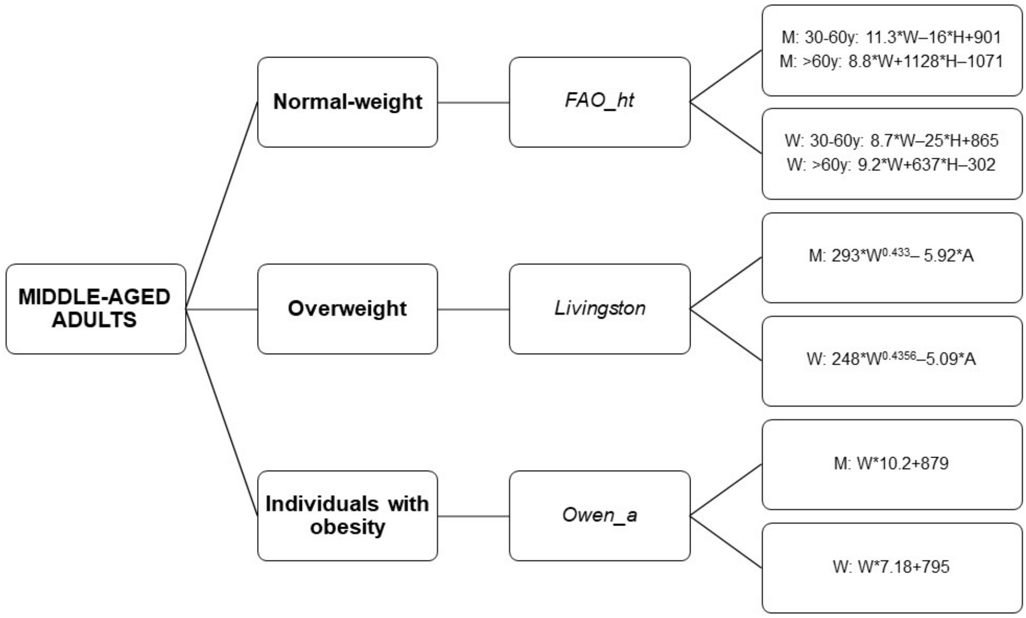

Figure 6. Decision tree to select a resting energy expenditure (REE) predictive equation by on weight status. "_a" refers to predictive equations which required only anthropometric parameters to calculate REE, and "_ht" refers to predictive equations which are proposed by the same author and include height. Abbreviations: M: Men; W: Women; W: Weight; H: Height; A: Age; y: years.

In individuals with obesity, the equation of Owen [17,18] showed the highest accuracy values (52.9% of prediction accuracy, mean absolute differences: 131 ± 137 Kcal), that concur with another study conducted in Australian individuals with obesity (~47 years of age, 41.8% of prediction accuracy at ±10% of accuracy level) [44]. However, a recent systematic review suggested that the equation of Mifflin [19] was the most accurate predictive equation for individuals with obesity (48% of prediction accuracy, applying an accuracy level of ±5%) [37], which differs from our results (23.5% of prediction accuracy). This difference might be partially explained by the inclusion of only 5 REE predictive equations in Frankenfield et al. [37]: Mifflin [19], Livingston [20], Harris Benedict [6], and FAO/WHO/UNU [22] equations. The Mifflin equation [19] has also been proposed as the most accurate REE predictive equation in Belgian women with obesity [43] (68% of accuracy at ±10% accuracy level), in Taiwanese individuals with obesity (46.3% of accuracy at ±10% accuracy level) [46], and in 1,900 Italian individuals with obesity (39.7% of accuracy at ±10% accuracy level).

We noted that the inclusion of body composition parameters (FM, FFM, or LM) did not improve the accuracy of the REE prediction in our participants. This is especially relevant because age, weight, and height-derived equations are more feasible in the clinical practice.

Limitations

The results of this study should be considered with caution: (i) our participants were middle-aged healthy sedentary adults (45-65 years of age), hence we cannot extend our results to older or younger individuals, (ii) although we did not find interaction by sex, our results need to be confirmed studying the role of sex and weight status together, (iii) although it is well known that metabolic carts can overestimate or underestimate the REE measure, it is important to consider that our data collection and the analysis process was strictly controlled and standardized, (iv) the respiratory exchange was measured using a neoprene facemask and not a canopy, as it is usually the case, and this issue could influence the results on the validity of equations used to estimate REE, (v) the low sample size in women with obesity and (vi) the use of the Weir equation implies assumptions that may not be accurate enough (e.g. absence of protein oxidation). Consequently, this could prevent us from extending our results to other populations with obesity, which present higher FM values compared to overweight populations.

CONCLUSIONS

In conclusion, our study shows that the REE predictive equation varies depending of the weight status in sedentary middle-aged adults. Future studies must be conducted in order to confirm the results obtained in older and younger individuals

REFERENCES

1 Smith KB, Smith MS. Obesity Statistics. *Prim Care Clin Off Pract* 2016; **43**: 121–135.

2 Fleming T, Robinson M, Thomson B, Graetz N, Margono C, Abraham J *et al.* Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study. *The Lancet* 2013; **384**: 766–81.

3 Noreik M, Maurmann M, Meier V, Becker I, Röhrig G, Polidori MC *et al.* Resting energy expenditure (REE) in an old-old population: implications for metabolic stress. *Exp Gerontol* 2014; **59**: 47–50.

4 Matarese L. Indirect Calorimetry. *J Am Diet Assoc* 1997; **97**: 154-S160.

5 Blasco Redondo R. Resting energy expenditure; assessment methods and applications. *Nutr Hosp* 2015; **31**: 245–254.

6 Harris J, Benedict F. A biometric study of basal metabolism in man. *Proc Natl Acad Sci* 1918; **4**: 370–373.

7 Roza AM, Shizgal HM. The Harris Benedict equation reevaluated: resting energy requirements and the body cell mass. *Am J Clin Nutr* 1984; **40**: 168–82.

8 Korth O, Bosy-Westphal A, Zschoche P, Glüer CC, Heller M, Müller MJ. Influence of methods used in body composition analysis on the prediction of resting energy expenditure. *Eur J Clin Nutr* 2007; **61**: 582–9.

9 Lorenzo A De, Andreoli A, Bertoli S, Testolin G, Oriani G, Deurenberg P. Resting metabolic rate in Italians : relation with body composition and anthropometric parameters. 2000; 77–81.

10 Johnstone AM, Rance KA, Murison SD, Duncan JS, Speakman JR. Additional anthropometric measures may improve the predictability of basal metabolic rate in adult subjects. *Eur J Clin Nutr* 2006; **60**: 1437–44.

11 Weijs PJM. Validity of predictive equations for resting energy expenditure in US and Dutch overweight and obese class I and II adults aged 18-65 y. *Am J Clin Nutr* 2008; **88**: 959–70.

12 Frankenfield DC, Rowe WA, Smith JS, Cooney RN. Validation of several established equations for resting metabolic rate in obese and nonobese people. *J Am Diet Assoc* 2003; **103**: 1152–9.

13 de la Cruz Marcos S, de Mateo Silleras B, Camina Martín MA, Carreño Enciso L, Miján de la Torre A, Galgani Fuentes JE *et al.* Proposal for a new formula for estimating resting energy expenditure for healthy spanish population. *Nutr Hosp* 2015; **32**: 2346–52.

14 Cunningham JJ. A reanalysis of the factors influencing basal metabolic rate in normal adults. *Am J Clin Nutr* 1980; **33**: 2372–4.

15 Huang K-C, Kormas N, Steinbeck K, Loughnan G, Caterson ID. Resting Metabolic Rate in Severely Obese Diabetic and Nondiabetic Subjects. *Obes Res* 2004; **12**: 840–845.

16 De Luis DA, Aller R, Izaola O, Romero E. Prediction equation of resting energy

expenditure in an adult spanish population of obese adult population. *Ann Nutr Metab* 2006; **50**: 193–196.

17 Owen OE, Holup JL, D'Alessio DA, Craig ES, Polansky M, Smalley KJ *et al.* A reappraisal of the caloric requirements of men. *Am J Clin Nutr* 1987; **46**: 875–85.

18 Owen OE, Kavle E, Owen RS, Polansky M, Caprio S, Mozzoli MA *et al.* A reappraisal of caloric requirements in healthy women. *Am J Clin Nutr* 1986; **44**: 1–19.

19 Mifflin MD, St Jeor ST, Hill LA, Scott BJ, Daugherty SA, Koh YO. A new predictive equation for resting energy expenditure in healthy individuals. *Am J Clin Nutr* 1990; **51**: 241–7.

20 Livingston EH, Kohlstadt I. Simplified resting metabolic rate-predicting formulas for normal-sized and obese individuals. *Obes Res* 2005; **13**: 1255–62.

21 Schofield WN. Predicting basal metabolic rate, new standards and review of previous work. *Hum Nutr Clin Nutr* 1985; **39 Suppl 1**: 5–41.

22 FAO/WHO/UNU. Energy and protein requirements. Geneva, Switzerland, 1985.

23 Henry CJK. Basal metabolic rate studies in humans: measurement and development of new equations. *Public Health Nutr* 2005; **8**: 1133–52.

24 Müller MJ, Bosy-Westphal A, Klaus S, Kreymann G, Lührmann PM, Neuhäuser-Berthold M *et al.* World Health Organization equations have shortcomings for predicting resting energy expenditure in persons from a modern, affluent population: generation of a new reference standard from a retrospective analysis of a German database of resting energy expe. *Am J Clin Nutr* 2004; **80**: 1379–90.

25 Song T, Venkataraman K, Gluckman P, Seng CY, Meng KC, Khoo EYH *et al.* Validation of prediction equations for resting energy expenditure in Singaporean Chinese men. *Obes Res Clin Pract* 2014; **8**: 201–208.

26 Siervo M, Bertoli S, Battezzati A, Wells JC, Lara J, Ferraris C *et al.* Accuracy of predictive equations for the measurement of resting energy expenditure in older subjects. *Clin Nutr* 2014; **33**: 613–619.

27 Acar-Tek N, Ağagündüz D, Çelik B, Bozbulut R. Estimation of Resting Energy Expenditure: Validation of Previous and New Predictive Equations in Obese Children and Adolescents. *J Am Coll Nutr* 2017; **36**: 470–480.

28 Wahrlich V, Teixeira TM, Anjos LA dos. Validity of a population-specific BMR predictive equation for adults from an urban tropical setting. *Clin Nutr* 2016.

29 Johannsen DL, Welk GJ, Sharp RL, Flakoll PJ. Differences in daily energy expenditure in lean and obese women: The role of posture allocation. *Obesity* 2008; **16**: 34–39.

30 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.

31 World Health Organization. Obesity: Preventing and managing the Global Epidemic. *Exec Summ WHO Tech Rep Ser* 2010; **1997**: 5–8.

32 Compher C, Frankenfield D, Keim N, Roth-Yousey L, Evidence Analysis Working Group. Best practice methods to apply to measurement of resting metabolic rate in adults: a systematic review. *J Am Diet Assoc* 2006; **106**: 881–903.

33 Fullmer S, Benson-Davies S, Earthman CP, Frankenfield DC, Gradwell E, Lee PSP *et al.* Evidence analysis library review of best practices for performing indirect calorimetry in healthy and non-critically ill individuals. *J Acad Nutr Diet* 2015; **115**: 1417–1446.e2.

34 Sanchez-Delgado G, Alcantara JMA, Ortiz-Alvarez L, Xu H, Martinez-Tellez B, Labayen I *et al.* Reliability of resting metabolic rate measurements in young adults: Impact of methods for data analysis. *Clin Nutr* 2017.

35 Weir J. New methods for calculating metabolic rate with special reference to protein metabolism. *J Physiol* 1949; **109**: 1–9.

36 Willis EA, Herrmann SD, Ptomey LT, Honas JJ, Bessmer CT, Donnelly JE *et al.* Predicting resting energy expenditure in young adults. *Obes Res Clin Pract* 2014; **8**: 201–208.

37 Frankenfield D. Bias and accuracy of resting metabolic rate equations in non-obese and obese adults. *Clin Nutr* 2013; **32**: 976–82.

38 Ruiz JR, Ortega FB, Rodríguez G, Alkorta P, Labayen I. Validity of resting energy expenditure predictive equations before and after an energy-restricted diet intervention in obese women. *PLoS One* 2011; **6**.

39 Frankenfield D, Roth-Yousey L, Compher C. Comparison of predictive equations for resting metabolic rate in healthy nonobese and obese adults: a systematic review. *J Am Diet Assoc* 2005; **105**: 775–89.

40 Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet (London, England)* 1986; **1**: 307–10.

41 De Lorenzo A, Tagliabue A, Andreoli A, Testolin G, Comelli M, Deurenberg P. Measured and predicted resting metabolic rate in Italian males and females, aged 18-59 y. *Eur J Clin Nutr* 2001; **55**: 208–14.

42 Lazzer S, Agosti F, Resnik M, Marazzi N, Mornati D, Sartorio A. Prediction of resting energy expenditure in severely obese Italian males. *J Endocrinol Invest* 2007; **30**: 754–61.

43 Weijs PJM, Vansant GAAM. Validity of predictive equations for resting energy expenditure in Belgian normal weight to morbid obese women. *Clin Nutr* 2010; **29**: 347–51.

44 Wright TG, Dawson B, Jalleh G, Guelfi KJ. Accuracy of resting metabolic rate prediction in overweight and obese Australian adults. *Obes Res Clin Pract* 2016; **10**: S74–S83.

45 Madden AM, Mulrooney HM, Shah S. Estimation of energy expenditure using prediction equations in overweight and obese adults: a systematic review. *J Hum Nutr Diet* 2016; **29**: 458–476.

46 Tseng C-K, Hsu H-S, Ho C-T, Huang H-Y, Liu C-S, Lin C-C *et al.* Predictive equation of resting energy expenditure in obese adult Taiwanese. *Obes Res Clin Pract* 2011; **5**: e313–e319.

RESULTS & DISCUSSION

SECTION 1: S-Klotho protein and physical fitness

This section includes a total of two chapters aiming to examine the association of body composition and physical fitness with S-Klotho in sedentary middle-aged adults. Concretely, it has been analized: [i] the association of LM and FM as well as BMD with S-Klotho (**Study 9**), and [ii] the association of sedentary, physical activity, and physical fitness levels (i.e. cardiorespiratory fitness and muscular strength) with S-Klotho in sedentary middle-aged adults (**Study 10**).

Chapter 5:
Body composition and S-Klotho in middle-aged adults: a cross-sectional study
(Study 9)

ABSTRACT

The a-Klotho gene was identified as a possible "ageing-suppressor" agent that extends lifespan when overexpressed. However, little is known about the association of the body composition with the secreted protein form of the a-Klotho gene (S-Klotho). Therefore, the aim of this study was to analyse the association of body composition including lean and fat mass as well as BMD with S-Klotho plasma levels in middle-aged sedentary adults.

A total of 74 (39 women) middle-aged sedentary adults (53.7±5.1 years old; 75.7±14.0 kg; 167.8±9.8 cm) participated in the study. We measured weight and height, and we used dual-energy X-ray absorptiometry to measure fat mass and lean mass. We calculated the BMI, FMI, and LMI. The S-Klotho plasma levels were measured in the EDTA plasma using a solid-phase sandwich enzyme-linked immunosorbent assay.

There was a strong positive association between LMI and S-Klotho plasma levels (β=74.794, R2=0.346, P<0.001), which persisted after controlling for age and sex as well as after additionally controlling for FMI. Significantly positive associations of BMI and BMD were also found with S-Klotho plasma levels (β=33.981, R2=0.125, P=0.002; and β=858.194, R2=0.058, P=0.041, respectively), which disappeared after controlling for LMI (β=0.183, R2=0.611, P=0.984; and β=-379.426, R2=0.617, P=0.290, respectively). FMI was not significantly associated with S-Klotho plasma levels.

Our study shows that LMI is strongly associated with S-Klotho plasma levels and explains the associations of BMI and BMD with S-Klotho plasma levels in middle-aged sedentary adults.

BACKGROUND

The ageing process is defined as the age-related deterioration of physiological functions necessary for the survival and fertility of an organism [1]. Nowadays, 8.5% of people world-wide (617 million) are over the age of 60. By 2050, this population will triplicate, and simultaneously the global lifespan is expected to increase by almost 8 years [2]. Therefore, it is necessary to find strategies to keep an optimal health during old age to prevent ageing from causing an important public health problem [2,3].

The human body composition changes dramatically during the ageing process, with decreases of the LM and the BMD, while the FM increases [4]. These body composition changes are also directly involved in several diseases related to the ageing process, such as sarcopenia [5], energy metabolism disorders and obesity [6], and/or osteoporosis [7], and may result in a decreased quality of life, an increased dependence, and an increased mortality risk in elderly population [8].

The α-Klotho gene was identified as a possible "ageing-suppressor" agent that accelerates ageing when disrupted and extends lifespan when overexpressed [9]. A defect in the α-Klotho gene causes multiple ageing-like phenotypes related to the body composition changes such as sarcopenia, energy metabolism disorders, obesity, and osteoporosis in mice [1,9,10]. In humans, it can be expressed as three different forms [11,12]: (i) the intra-cellular form, which binds Na-ATPase, (ii) the cell-membrane form, which creates a complex with FGF23 and FGFR1, and (iii) S-Klotho, identified in blood, plasma, urine, and cerebrospinal fluid. The S-Klotho reliably indicate the α-Klotho gene expression, which also progressively decrease in humans after 40 years of age [13].

Little is known about the association of the body composition with S-Klotho in humans. The results of previous studies have shown that the skeletal muscle contraction could modulate the α-Klotho gene expression [14]. Furthermore, a positive strong association of muscular strength [15] and functioning [16] with S-Klotho have been reported, but, to the best of our knowledge, the relationship between the LM and the S-Klotho has not been studied in humans previously. Similarly, whether body fatness is associated with S-Klotho is unknown. Controversial findings have been published regarding the association of the BMD and the S-Klotho. Cross-sectional studies have reported that a lower BMD is associated with α-Klotho gene deficiency [17,18], whereas a longitudinal study suggested that the BMD was unrelated to the S-Klotho in older adults [19]. In addition, understanding whether body composition parameters are associated with S-Klotho in adults is of clinical interest.

Therefore, we analysed the association between body composition including lean and FM as well as BMD with S-Klotho in sedentary middle-aged adults.

MATERIAL & METHODS

Participants

A total of 74 (39 women) sedentary middle-aged adults (45-65 years old) participated in the present study. The participants were enrolled in the FIT-AGEING study [20], an exercise-based randomised controlled trial (clinicaltrial.gov: ID: NCT03334357). The study was approved by the Human Research Ethics Committee of the "Junta de Andalucía" [0838-N-2017]. All participants received a comprehensive preventive medical examination, were sedentary (<20 min physical activity on <3 days/week), did not smoke or take any medication, had a stable weight in the last 3 months (<3 kg change), and had a normal electrocardiogram. The evaluations were performed between September and October 2015 and 2016 at the *Centro de Investigación Deporte y Salud (CIDS)* and at the "Campus de la Salud" Hospital. The study protocols and design were applied in accordance with the revised ethical guidelines of the Declaration of Helsinki. All participants signed an informed consent.

Body composition assessment

The weight and height measurements were performed without shoes and with light clothing, using a pre-validated scale and stadiometer (Seca 760, Electronic Column Scale, Hamburg, Germany). A dual-energy X-ray absorptiometry scanner (Discovery Wi, Hologic, Inc., Bedford, MA, USA) was used to measure the LM (kg), the FM (kg), and the BMD (g/cm^2). The whole-body scan was considered to obtain all body composition parameters. We conducted the quality controls, the positioning of the participants, and the analyses of the results following the manufacturer's recommendations. An automatic delineation of the anatomic regions was performed by the software APEX 4.0.2. We acquired spine phantom quality control scans on each study day.

We calculated the BMI as weight in kg divided by height in meters^2 and the LMI as LM in kg divided by height in meters^2. Similarly, we calculated the FMI as FM in kg divided by height in meters^2. FM was also expressed as percentages of total body mass. The participants were categorised as normal-weight (BMI ≥18.5 and <25 kg/m^2), overweight (BMI ≥25 and <30 kg/m^2), and obese (BMI ≥30 kg/m^2).

S-Klotho assessment

The blood samples were collected from the antecubital vein in the morning after fasting for 12 hours. The S-Klotho was measured in the EDTA plasma using a solid-phase sandwich ELISA (Demeditec, Kiel, Germany). The kit used two types of highly specific antibodies, and its optical density was measured at a wavelength of 450 nm ± 2 nm. All participants were requested to abstain from drugs and/or caffeine, to eat a standardised dinner before sampling, and to avoid any physical activity of moderate (24 hours before) and/or vigorous intensity (48 hours before).

Statistical analysis

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 SD. We performed the T-Student unpaired-samples test to study differences between men and women. We conducted simple linear regression models (Model 1) to examine the association of body composition (i.e. BMI, LMI, FMI, and BMD) with levels of S-Klotho. We also conducted multiple linear regression models to test these associations after adjusting by age and sex as well as by LMI or FMI where appropriate. We performed an ANOVA to compare S-Klotho across weight status (normal-weight, over-weight, and obese) and an ANCOVA to test differences of S-Klotho across weight status adjusting for LMI followed by the Bonferroni post-hoc test. No interaction by sex was observed ($P>0.05$), hence the appropriateness of fitting models for men and women was combined, with sex entered as a covariable. The analyses were conducted using the Statistical Package for Social Sciences (IBM Corporation, Chicago, IL, USA), and the level of significance was set at <0.05.

RESULTS

The baseline characteristics of the participants by sex are shown in Table 1. S-Klotho was similar in men and women ($P=0.398$). Figure 1 shows the association between body composition parameters and S-Klotho. There was a significant positive association between BMI and S-Klotho ($\beta=33.981$, $R^2=0.125$, $P=0.002$; Figure 1A), which persisted after including sex and age in the model ($\beta=35.591$, $R^2=0.136$, $P=0.004$). However, this association disappeared once LMI was included in the model ($\beta=0.183$, $R^2=0.611$, $P=0.984$). We observed a strong positive association between LMI and S-Klotho ($\beta=74.794$, $R^2=0.346$, $P<0.001$; Figure 1B), which persisted after controlling for age and sex ($\beta=147.858$, $R^2=0.611$, $P<0.001$) as well as after additionally controlling for FMI ($\beta=147.726$, $R^2=0.611$, $P<0.001$).

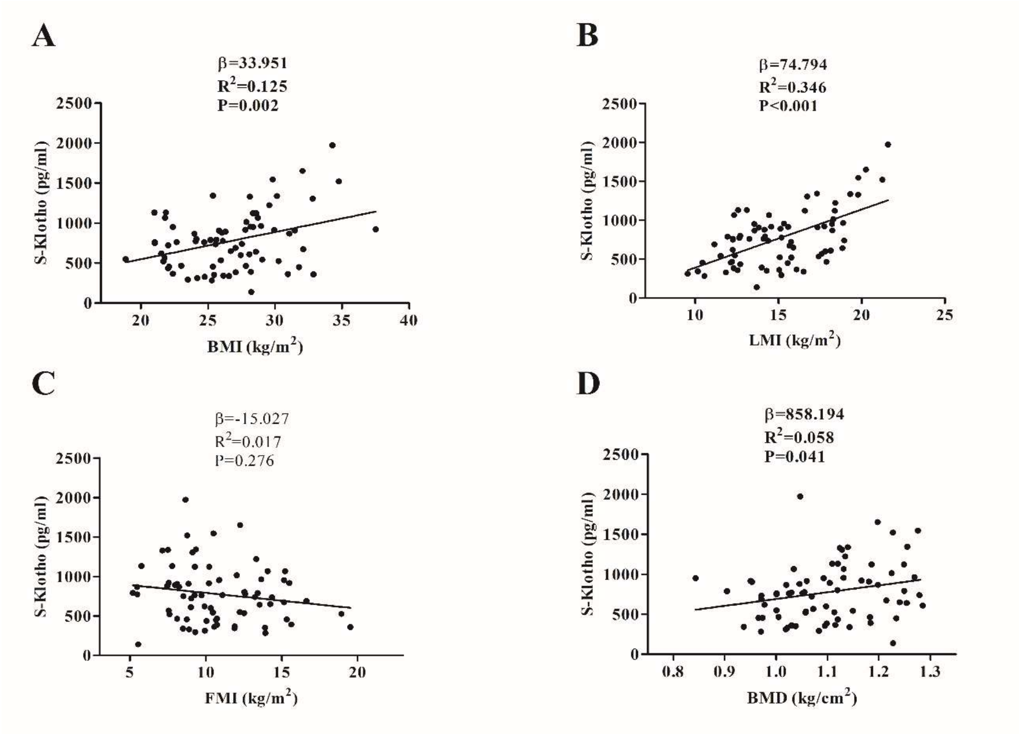

Figure 1. Association between the body composition variables which include the body mass index (BMI, Panel A), the lean mass index (LMI, Panel B), the fat mass index (FMI, Panel C), and the bone mineral density (BMD, Panel D) with S-Klotho in middle-age sedentary adults. β (unstandardized regression coefficient), R2, and P from a simple linear regression analysis.

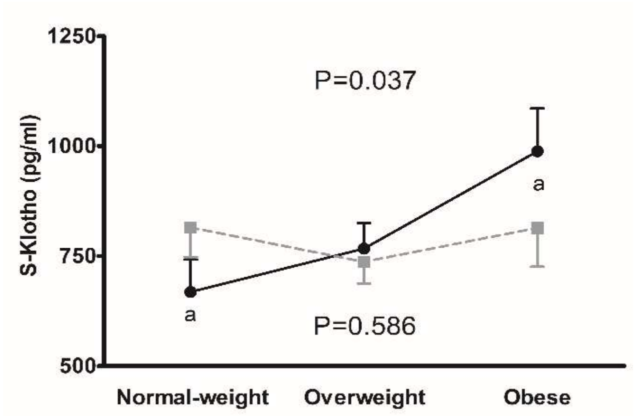

Figure 2. S-Klotho by the weight status categories in middle-age adults (black lines), S-Klotho (adjusted by LMI) by the weight status in middle-age adults (grey lines). Values are presented as means and 95% confidence interval. Repeated letters (a-a; b-b, etc.) indicate P<0.05: ANOVA to compare the S-Klotho across weight status (normal-weight, over-weight, and obese), and ANCOVA to compare the S-Klotho levels across weight status after adjusting by LMI followed by Bonferroni post-hoc test.

FMI was not significantly associated with S-Klotho (β=-15.027, R^2=0.017, P=0.276; Figure 1C). There was a positive association between BMD and S-Klotho (β=858.194, R^2=0.058, P=0.041; Figure 1D) which was attenuated once sex and age was included in the model (β=908.897, R^2=0.065, P=0.081) and disappeared after additionally controlling for LMI (β=-379.426, R^2=0.617, P=0.290).

Figure 2 shows S-Klotho across weight status categories before and after adjusting for LMI. Obese people had higher levels of S-Klotho compared with their normal-weight counterparts (988.08 vs. 668.43 pg/ml, P=0.033, Figure 2), yet these differences disappeared once the analyses were adjusted for LMI (816.44 vs. 809.59, P=1.000, Figure 2). The results remained after further adjusting for sex or age. We repeated all the analyses in men and women separately, and the pattern of the association did not change.

DISCUSSION

The present study shows that LMI is strongly associated with S-Klotho in sedentary middle-aged adults. We observed that the association between BMI and BMD with S-Klotho disappeared once LMI was accounted for. To the best of our knowledge, this is the first study to show the relationship between LMI and S-Klotho in adults.

These novel findings suggest that, in addition to the reported positive strong association of the muscular strength measured by grip strength [15] and muscle function measured by daily living activities [16] with S-Klotho, LMI could be an excellent predictor of S-Klotho in adults. Several mechanisms could explain these findings: (i) The alteration of the α-Klotho gene expression is associated with a decreased stem cell frequency [14,21,22], impaired angiogenesis [23], and decreased cellular resistance to stress [22,24]. The physiological mechanism that explains this association could be based on the inhibition of the Wnt signalling activation (which induces a subsequent inactivation of fibrogenic signalling pathways) derived from high α-Klotho gene expression [21,25], (ii) The α-Klotho gene is capable of inhibiting the TGF-β1 by binding the TGF-β1 receptor [26]. The TGF-β1 is considered as "master switch" for promoting mesenchymal transition toward a fibroblastic lineage in several tissues [14,26]. Given this interaction, it is possible that age-related declines in S-Klotho may result in a decreased opposition of the TGF-β1 signalling, ultimately promoting fibrosis formation and impairing myofiber regeneration [26]. All of these facts are related to the sarcopenia process which is characterised by a lower skeletal muscle quantity, a lower muscular strength, and a lower muscular functioning. Therefore, the LMI (as an excellent index of muscle mass and muscle function) could play an important role in the α-Klotho gene expression and in S-Klotho independently of sex, age, and FMI.

We observed a lack of association of BMI and FMI with S-Klotho after taking LMI into account. The α-Klotho gene is involved in glucose control [27], phosphate metabolism [28], and diabetes mellitus [29], all of which are related to fat metabolism. We observed that LMI explained the observed association between BMI and S-Klotho. Moreover, we observed no differences in S-Klotho by weight status categories once we accounted for LMI. Our results do not concur with those findings reported by Amitami et al. [30]. They showed that S-Klotho were markedly lower in 12 obese women compared with 11 normal-weight women. However, this study considered neither LM nor FM, parameters which may play an important role in the energy metabolism and obesity (as our results show).

Chalhoub et al. [19] reported no association between the BMD and S-Klotho in a longitudinal cohort of 2,776 community-dwelling adults. These results concur with our findings when we included sex, age, and LMI as covariates. In contrast, some studies suggested that the α-Klotho gene expression could be involved in the bone metabolism in men [18] and women [17]. More studies that measure the cell-membrane form of the α-Klotho gene (not measured in our study) are needed to elucidate whether BMD is related to the α-Klotho gene expression, since the cell-membrane form is the bone derived hormone which is thought to be involved in bone regulation because of its coupling with FGF-23 [12].

Limitations

This study´s findings should, however, be taken with caution as some limitations arise. Firstly, as our study is observational, no causal relationship can be established. Secondly, the relatively small sample size and the fact that our study only included sedentary middle-aged adults (45-65 years old), and hence we cannot extend the results to older, younger, and/or physically active individuals.

CONCLUSIONS

In summary, our study shows that LMI is strongly associated with S-Klotho, and it explains the associations observed of the BMI and BMD with S-Klotho in sedentary middle-aged adults. Moreover, our data support the notion that the skeletal muscle tissue plays an important role in S-Klotho metabolism, or vice versa. Nonetheless, further studies are needed to confirm the observed association in older, younger, physically active individuals, and to establish whether the body composition plays a role on the intra-cellular form and on the cell-membrane form of the α-Klotho gene. Intervention studies are needed to understand whether changes in LMI are associated with changes in S-Klotho.

Perspectives

We show, for the first time, that LMI is strongly associated with S-Klotho in middle-aged adults independently of sex, age, and FM. Considering S-Klotho as an excellent ageing biomarker, our results support the idea that maintaining an adequate LM level, the ageing process can be attenuated. Whether changes overtime in LMI are associated with changes in S-Klotho or vice versa is now known and further studies are needed to confirm the observed association in older, younger, and physically active individuals, and to establish whether the body composition plays a role on the intra-cellular form and on the cell- membrane form of the α-Klotho gene. Exercise-based intervention studies aiming at improving LM are needed to better understand the role of body composition on S-Klotho.

REFERENCES

1 Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T *et al.* Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* 1997; **390**: 45–51.

2 Yasobant S. Comprehensive public health action for our aging world: the quintessence of public health policy. *J Int Med Res* 2018.

3 Pickering C, Kiely J. ACTN3, Morbidity, and Healthy Aging. *Front Genet* 2018; **9**: 15.

4 Kohara K. Sarcopenic obesity in aging population: current status and future directions for research. *Endocrine* 2014; **45**: 15–25.

5 Hirschfeld HP, Kinsella R, Duque G. Osteosarcopenia: where bone, muscle, and fat collide. *Osteoporos Int* 2017; **28**: 2781–2790.

6 Frasca D, Blomberg BB, Paganelli R. Aging, obesity, and inflammatory age-related diseases. *Front Immunol* 2017; **8**: 1–10.

7 Sugiyama T, Oda H. Osteoporosis therapy: Bone modeling during growth and aging. *Front Endocrinol (Lausanne)* 2017; **8**: 8–11.

8 Hamer M, O'Donovan G. Sarcopenic obesity, weight loss, and mortality: the English Longitudinal Study of Ageing. *Am J Clin Nutr* 2017; **106**: 125–129.

9 Kuro-o M. Klotho. *Pflugers Arch Eur J Physiol* 2010; **459**: 333–343.

10 Kawaguchi H, Manabe N, Miyaura C, Chikuda H, Nakamura K, Kuro-o M. Independent impairment of osteoblast and osteoclast differentiation in klotho mouse exhibiting low-turnover osteopenia. *J Clin Invest* 1999; **104**: 229–237.

11 Imura A, Iwano A, Tohyama O, Tsuji Y. Secreted Klotho protein in sera and CSF: implication for post-translational cleavage in release of Klotho protein from cell membrane. *FEBS Lett* 2004; **565**: 143–147.

12 Kim J-H, Hwang K-H, Park K-S, Kong ID, Cha S-K. Biological role of anti-aging protein Klotho. *J lifestyle Med* 2015; **5**: 1–6.

13 Saghiv MS, Sira D Ben, Goldhammer E, Sagiv M. The effects of aerobic and anaerobic exercises on circulating soluble-Klotho and IGF-I in young and elderly adults and in CAD patients. *J Circ Biomarkers* 2017; **6**: 1–8.

14 Avin KG, Coen PM, Huang W, Stolz DB, Sowa GA, Dubé JJ *et al.* Skeletal muscle as a regulator of the longevity protein, Klotho. *Front Physiol* 2014; **5**: 189.

15 Semba RRD, Cappola ARA, Sun K, Bandinelli S, Dalal M, Crasto C *et al.* Relationship of low plasma klotho with poor grip strength in older community-dwelling adults: the InCHIANTI study. *Eur J Appl Physiol* 2012; **112**: 1215–20.

16 Crasto C, Semba R, Sun K, Cappola A, Bandinelli S, Ferrucci L. Relationship of low-circulating "anti-aging" klotho hormone with disability in activities of daily living among older community-

dwelling adults. *Rejuvenation Res* 2012; **5**: 295–301.

17 Yamada Y, Ando F, Niino N, Shimokata H. Association of polymorphisms of the androgen receptor and klotho genes with bone mineral density in Japanese women. *J Mol Med* 2005; **83**: 50–57.

18 Zarrabeitia MT, Hernández JL, Valero C, Zarrabeitia AL, Ortiz F, Gonzalez-Macias J *et al.* Klotho gene polymorphism and male bone mass. *Calcif Tissue Int* 2007; **80**: 10–14.

19 Chalhoub D, Marques E, Meirelles O, Semba RD, Ferrucci L, Satterfield S *et al.* Association of Serum Klotho with Loss of Bone Mineral Density and Fracture Risk in Older Adults. *J Am Geriatr Soc* 2016; **64**: e304–e308.

20 Storlien L, Oakes ND, Kelley DE. Metabolic flexibility. *Proc Nutr Soc* 2004; **63**: 363–8.

21 Liu H, Fergusson MM, Castilho RM, Liu J, Cao L, Chen J *et al.* Augmented Wnt signaling in a mammalian model of accelerated aging. *Science* 2007; **317**: 803–6.

22 Izbeki F, Asuzu DT, Lorincz A, Bardsley MR, Popko LN, Choi KM *et al.* Loss of Kit low progenitors , reduced stem cell factor and high oxidative stress underlie gastric dysfunction in progeric mice. 2010; **16**: 3101–3117.

23 Fukino K, Suzuki T, Saito Y, Shindo T, Amaki T, Kurabayashi M *et al.* Regulation of angiogenesis by the aging suppressor gene klotho. 2002; **293**: 332–337.

24 Yamamoto M, Clark J, JV P, Gurnani P, Nandi A, Kurosu H. Regulation of oxidative stress by the anti-aging hormone klotho. *Biol Chem* 2005; **280**: 3829–3824.

25 Zhou L, Li Y, Zhou D, Tan RJ, Liu Y. Loss of Klotho contributes to kidney injury by depression of Wnt/catenin signaling. *J Am Soc Nephrol* 2013; **24**: 771–785.

26 Doi S, Zou Y, Togao O, Pastor J, John G, Wang L. Klotho inhibits transforming growth factor-beta1 (TGF-beta1) signaling and suppresses renal fibrosis and cancer metastasis in mice. *J Biol Chem* 2011; **286**: 8655–65.

27 Donate-Correa J, Martín-Núñez E, Delgado NP, de Fuentes MM, Arduan AO, Mora-Fernández C *et al.* Implications of Fibroblast growth factor/Klotho system in glucose metabolism and diabetes. *Cytokine Growth Factor Rev* 2016; **28**: 71–77.

28 Kuro-O M. Klotho, phosphate and FGF-23 in ageing and disturbed mineral metabolism. *Nat Rev Nephrol* 2013; **9**: 650–660.

29 Nie F, Wu D, Du H, Yang X, Yang M, Pang X *et al.* Serum klotho protein levels and their correlations with the progression of type 2 diabetes mellitus. *J Diabetes Complications* 2017; **31**: 594–598.

30 Amitani M, Asakawa A, Amitani H, Kaimoto K, Sameshima N, Koyama K *et al.* Plasma klotho levels decrease in both anorexia nervosa and obesity. *Nutrition* 2013; **29**: 1106–1109.

Chapter 6:
Association of physical activity and fitness with S-Klotho in sedentary middle-aged adults: The FIT-AGEING study
(Study 10)

ABSTRACT

The aim of the current study was to determine the association of sedentary, physical activity, and physical fitness levels (i.e. cardiorespiratory fitness and muscular strength) with the shed form of the a-Klotho gene (S-Klotho plasma levels) in middle-aged sedentary adults.

A total of 74 (52.7% women) middle-aged sedentary adults (53.7±5.1 years old) were enrolled in the FIT-AGEING study. Physical activity and sedentary time were assessed with a wrist-worn accelerometer. VO_2max was determined by a maximum treadmill test using indirect calorimetry. Lower and upper body muscular strength were assessed by an isokinetic strength test and by the hand grip strength test, respectively. The S-Klotho plasma levels were measured in the EDTA plasma using a solid-phase sandwich enzyme-linked immunosorbent assay.

Based on the principal-component analysis, overall physical activity, moderate-vigorous physical activity levels, and sedentary time (as outcomes included in the sedentary time and physical activity category) explained a total of 17.5% of the cumulative variance in S-Klotho plasma levels, whereas extension peak torque, hand grip strength, and maximal oxygen uptake (as outcomes included in the physical fitness category) explained a total of 15.5% of the cumulative variance in S-Klotho plasma levels. Based on the loading of variables on these 2 categories, the percentage of cumulative variance explained was 28.9% of the S-Klotho plasma levels being higher (33.0% of cumulative variance) when sex was included in the model.

In summary, our results indicate that physical activity and physical fitness levels are associated with S-Klotho plasma levels in middle-aged sedentary adults.

BACKGROUND

Ageing is a complex and multifactorial process influenced by both genetic and environmental factors, and characterised by a progressive decline of physiological functions, which leads to an impaired physical integrity and an increase of mortality risk [1,2]. There are a number of age-related diseases including metabolic and cardiovascular diseases, bone disorders, neurodegenerative diseases, or cancer, among others [3,4]. The ageing of the global population has progressively increased over the past decades, hence the incidence of age-related diseases has also increased causing a public health and economic burden [3,4]. To attenuate this problem, several proposals have been made, including pharmacological [5], nutritional [6], and physical activity [7] interventions.

The importance of physical activity for health and well-being during the ageing process is evident [8,9], since it has consistently been associated with better quality of life and life expectancy, lower incidence of ageing-related diseases, and a reduced risk of all-cause mortality [10–12]. Furthermore, a recent study that included over 1 million adults reported that high levels of sedentary time increased premature cardiovascular and all-cause mortality risk, regardless of physical activity levels [13]. Exercise (understood as programmed physical activity) is considered a highly effective form of promoting healthy ageing through physical fitness improvements [14,15], since the main components of physical fitness (cardiorespiratory fitness and muscular strength) are powerful predictors of longevity and both cardiovascular and all-cause mortality [16–20]. Therefore, identifying the factors that play a role in the association of physical activity and physical fitness with health improvements during the ageing process is crucial for understanding ageing physiology.

The α-Klotho gene was identified in 1997 [21] as a mutated gene in transgenic mice. It extends lifespan when overexpressed and accelerates ageing-like phenotypes when disrupted (e.g. sarcopenia, impaired cognition, atherosclerosis, endothelial dysfunction, impaired mineral metabolism, osteoporosis, growth retardation, hypokinesis and gait disturbance, skin atrophy, and emphysema) [21–24]. The α-Klotho gene encodes a single-pass transmembrane glycoprotein expressed predominantly in the distal tubule cells of the kidney, parathyroid glands, and choroid plexus of the brain. There are three protein domains [21,24–28]: (i) the intracellular domain, the functions of which are not yet fully understood [25], (ii) the extracellular domain, which acts as an obligate co-receptor of FGF23 (a bone-derived hormone that regulates phosphate excretion) [25] and holds a potential site for proteolytic cleavage [27,28], and (iii) S-Klotho, obtained via alternative splicing and identified in plasma, cerebrospinal fluid, and urine [25,26]. In humans, S-Klotho accurately indicate the α-Klotho gene expression, hence the S-Klotho have been established as an excellent anti-ageing biomarker [29]. It was previously suggested that S-Klotho is independently associated with a lower likelihood of having cardiovascular diseases [30] and that it is an independent predictor of all-cause mortality [31].

Although some studies have suggested a significant increase of S-Klotho in response to a single bout of exercise in healthy individuals [32,33] and after 3 months of low-to-moderate intensity aerobic training in postmenopausal women [34], little is known about the association of physical activity and physical fitness with S-Klotho. A study showed a positive association between muscular strength and S-Klotho in mice [35] and in individuals over the age of 70 [36,37]. However, whether physical activity and physical fitness are associated with S-Klotho in middle-aged adults (aged from 45-65 years old) is unknown. Considering that the problem with studying ageing in the elderly is that many of them already have age-related diseases [3,4], it has been previously suggested that interventions to reverse or delay age-related diseases must take place when individuals are still healthy and relatively young [38,39].

We determined the association of sedentary, physical activity, and physical fitness levels (i.e. cardiorespiratory fitness and muscular strength) with S-Klotho in sedentary middle-aged adults.

MATERIAL & METHODS

A total of 74 (52.7% women) sedentary middle-aged adults (aged from 45 to 65 years old) were enrolled in the present study. The participants were involved in the FIT-AGEING study [40], an exercise-based randomised controlled trial (clinicaltrial.gov: ID: NCT03334357). This study was approved by the Human Research Ethics Committee of the "Junta de Andalucía" [0838-N-2017]. Before the evaluations (conducted in September-October 2015 and in September-October 2016 at the *Centro de Investigación Deporte y Salud (CIDS)*, Granada, Spain), the participants signed an informed consent. The study design followed the revised ethical guidelines of the Declaration of Helsinki (last revision). The inclusion criteria were (i) being sedentary, (ii) being a non-smoker, (iii) not taking any medication, (iv) not having any acute or chronic illness, (v) having had a stable weight in the previous 3 months (<3 kg change), and (v) not being pregnant.

Physical activity and sedentary time and assessment

Physical activity and sedentary time were assessed with a wrist-worn accelerometer (ActiGraph GT3X+, Pensacola, FL, US) during 7 consecutive days (24 hours/day) [40]. The accelerometers were provided to the participants along with the instructions about how to wear it. They were also reminded to remove the accelerometer in water-based activities. 100 Hz were selected as the sampling frequency to store raw accelerations [41]. The ActiLife v.6.13.3 software (ActiGraph, Pensacola, FL, US) was used to export and convert raw data to ".csv" format. Afterwards, these files were processed with the GGIR package (v. 1.5-12, https://cran.r-project.org/web/packages/GGIR/) in R (v. 3.1.2, https://www.cran.r-project.org/). In short, the processing methods included a local gravity data auto-calibration [42], a determination of the Euclidean Norm Minus One, a calculation of non-wear time based on the raw acceleration of the three axes, the identification of malfunctioning of the accelerometer based on abnormal high accelerations, imputation of non-wear time and abnormal high accelerations, calculation of waking and sleeping time by an automatised algorithm guided by the participants' daily reports [43], and the determination of sedentary time, LPA, MPA, VPA, and MVPA using age-specific cut-points for Euclidean Norm Minus One [44,45]. The participants who did not wear the accelerometers for at least 16 hours/day during 4 days (including 1 weekend day) were excluded from the analysis.

Cardiorespiratory fitness assessment

VO_2max was determined using a maximum treadmill (H/P/Cosmos Pulsar treadmill, H/P/Cosmos Sport & Medical GMBH, Germany) exercise test following the modified Balke protocol, which has been extensively validated [46]. In short, the warm-up consisted in walking at 3 km/h for 1 minute followed by 2 minutes at 4 km/h. The incremental protocol started at a speed of 5.3 km/h (0% grade), which was kept constant with the gradient increasing by 1% every minute until the participants reached their volitional exhaustion. Thereafter, the participants underwent a cooling-down period (4 km/h and 0% grade for 5 minutes). VO_2 and VCO_2 were obtained by IC (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 at the beginning of every testing day, and the gas analyser was calibrated using two standard gas concentrations following the manufacturer's instructions before each test. The gas exchange parameters were averaged every 5 seconds with the Breeze Suite software (version 8.1.0.54 SP7, MGC Diagnostic®). In all assessments, the participants were strongly motivated to invest their maximum effort. Before the test, a familiarisation process with the 6–20 Borg scale [47] was conducted, which was used to measure the RPE during the last 15 seconds of each stage and at exhaustion. Heart rate was recorded every five seconds (Polar RS300, Kempele, Finland). We also registered gas exchange parameters, RPE, and heart rate during the cooling-down period. The criteria for achieving VO_2max were (i) RER ≥ 1.1, (ii) a plateau in VO_2 (changes of <100 ml/min in the last 60 seconds of the test), (iii) and a heart rate within 10 beats/min of the age-predicted maximal heart rate (209–0.73 ∗ age) [48]. We considered the peak oxygen uptake value during the maximum treadmill exercise test when these criteria were not met [48].

All participants were requested to abstain from caffeine (24 hours before), to fast for 3 hours eating a complete meal just before, and to avoid any physical activity of moderate (24 hours before) and/or vigorous intensity (48 hours before).

Muscular strength assessment

We determined the lower body muscular strength assessment on a different day (separated by 3-7 days) applying the same preconditions as in the cardiorespiratory fitness assessment. An isokinetic strength test was performed using a Gymnex Iso-2 dynamometer (EASYTECH s.r.l., Italy), calibrated following the manufacturer's instructions before the data collection. The knee flexor and extensor muscles were tested concentrically at 60° s^{-1}. The upper members, hips, and shoulders were stabilised with safety belts. The rotational axis of the dynamometer was aligned with the right lateral femoral condyle. The force pad was placed 3–4 cm above the medial malleolus. The knee extension was initiated at a joint angle of 90° and ended at 170°. The participants were instructed to submaximally flex and extend their knee five times, and then to complete three maximal repetitions. We allowed the participants a 1-minute rest between submaximal and maximal trials in accordance with a previously validated protocol [49]. The flexion and extension peak torque were determined as the single repetition with the highest muscular force output (Nm). The participants were strongly motivated during the test, and the same trained researcher conducted all the isokinetic tests. The intraclass correlation coefficient for test–retest reliability for this test was > 0.90 [50].

Hand grip strength was measured using a digital hand dynamometer (TKK 5101 Grip-D; Takey, Tokyo, Japan), and the scores were recorded in kilograms. The reported precision of the dynamometer was 0.1 kg. Two measurements were taken for each hand (right and left alternatively), with a 1-minute rest between trials. The participants were instructed to squeeze gradually and continuously for at least 2 seconds and were encouraged to do their best when performing the tests. The grip span of the dynamometer was fixed at 5.5 cm for men. For women, an adjustment to the individual's hand size was made, following a previous validated equation [51].

Body composition assessment

Weight (±10 g) and height (±0.1 cm) were assessed using a digital integrating scale and a stadiometer (Seca 760, Electronic Column Scale, Hamburg, Germany). BMI was calculated as weight (kg)/height $(m)^2$. We determined FM and LM by Dual Energy X-ray Absorptiometry (DXA, HOLOGIC, Discovery Wi).

S-Klotho plasma assessment

Blood samples were collected from the antecubital vein in the morning after an overnight fasting, 3-7 days before the physical fitness tests. A solid-phase sandwich ELISA (Demeditec, Kiel, Germany) was used to determine the S-Klotho in the EDTA plasma [29]. The kit used two types of highly specific antibodies, and its optical density was measured at a wavelength of 450 nm ± 2 nm. All participants were requested to abstain from drugs and/or caffeine, to eat a standardised dinner before the blood sample collection, and to refrain from any physical activity of moderate (24 hours before) and/or vigorous intensity (48 hours before).

Statistical analysis

Normal distribution of all variables was checked with the Shapiro-Wilk test, visual inspection of histograms, Q-Q, and box plots. The descriptive parameters are expressed as mean and SD. We performed the T-Student unpaired-samples test to study differences between men and women. A PCA was performed to quantify the dimensions supposed to underlie S-Klotho on a variety of outcomes and to reduce the initial set of variables, while checking for multicollinearity. We found collinearity between LM and all muscular strength-related parameters (Durbin-Watson coefficient≥1.706), thus we organized the study outcomes in two broad categories: (i) sedentary time and physical activity (including sedentary time, LPA, MPA, VPA, MVPA, overall physical activity), and (ii) physical fitness (VO_2max, flexion peak torque, extension peak torque, and hand grip strength).

A multiple-regression analysis was conducted to determine the explained variance in S-Klotho using the components derived from PCA. We also conducted a multiple-regression analysis including sex as covariates. The analyses were conducted using the Statistical Package for Social Sciences (SPSS, v. 22.0, IBM Corporation, Chicago, IL, USA), and the level of significance was set at <0.05.

RESULTS

Table 1 shows the baseline characteristics of the participants by sex. No statistically significant differences were observed in S-Klotho between men and women (P=0.4).

From PCA analysis, 3 components for the sedentary time and physical activity category, and 3 components for the physical fitness category, were extracted and labelled in the following order: (i) overall physical activity, (ii) MVPA, and (iii) sedentary time for the sedentary time and physical activity category, and (i) extension peak torque, (ii) hand grip strength, and (iii) VO_2max for the physical fitness category. The outcomes were well defined, and their communalities ranged from 0.81 to 0.97 in the sedentary time and physical activity category, and ranged from 0.68 to 0.87 in the physical fitness category. Moreover, based on the loading of variables on the 3 components, the percentage of cumulative variance explained was 17.5 % for the sedentary time and physical activity category, and 15.5% for the physical fitness category, of S-Klotho, respectively.

Figure 1 shows the association between outcomes included in both sedentary time and physical activity, and the physical fitness categories with S-Klotho in sedentary middle-aged adults. No association was found between sedentary time and overall physical activity with S-Klotho ($P \geq 0.2$, Figures 1A and 1E), whereas a positive association between MVPA and S-Klotho was observed ($P=0.011$, Figure 1C) in our study participants. We found a positive association between VO_2max, extension peak torque, and hand grip strength with S-Klotho (all $P \leq 0.003$, Figure 1B, 1D, and 1 F, respectively).

A multiple regression analysis was performed to determine the variance explained in S-Klotho by the categories obtained by PCA analysis (i.e. sedentary time and physical activity, and the physical fitness categories). Correlation coefficients between these outcomes and the S-Klotho revealed that MVPA, hand grip strength, and VO_2max were significantly related to performance ($R=0.50$, $R=0.36$, $R=0.34$; $P<0.001$). Based on the loading of variables on these 2 components, the percentage of cumulative variance explained was 28.9% of S-Klotho (Table 2). The results persisted when sex was included in the model as a covariate, being 33.0% the percentage of cumulative variance explained of S-Klotho (Table 2).

DISCUSSION

The main finding of this study is that physical activity and fitness levels are associated with S-Klotho in sedentary middle-aged adults.

Physical activity represents a cornerstone in the preservation of health and well-being during the senescence process and in the primary prevention of at least 35 ageing-related chronic diseases [8,9]. Indeed, physical activity levels are strongly associated with higher quality of life and longevity, lower prevalence of ageing-related chronic diseases, and a decreased risk of all-cause mortality [10-12]. S-Klotho could be a key factor modulating the above-mentioned relationship, since previous studies conducted in older adults suggested that higher S-Klotho is associated with activities of daily living ability [52] and lower likelihood of having cardiovascular disease [30].

Our results suggest a positive association between MVPA and S-Klotho, whereas no association was observed between LPA and S-Klotho in sedentary middle-aged adults. Therefore, increasing physical activity intensity (LPA to MVPA) could exert a significant effect on S-Klotho in sedentary middle-aged adults [53].

Table 1. Descriptive characteristic of participants.

	N	All		N	Men		N	Women	
Age (years)	74	53.7	(5.1)	35	54.4	(5.3)	39	53.0	(5.0)
S-Klotho (pg/ml)	73	775	(364)	34	814	(452)	39	741	(266)
Body composition									
BMI (kg/m^2)	74	26.7	(3.8)*	35	28.3	(3.6)	39	25.3	(3.3)
FM (%)	74	39.9	(9.1)*	35	34.7	(8.0)	39	44.5	(7.4)
FM (kg)	74	30.0	(8.4)	35	30.9	(9.8)	39	29.2	(7.1)
LM (kg)	74	43.5	(11.7)*	35	53.9	(6.5)	39	34.1	(5.8)
Physical activity									
Sedentary time (min/day)	71	745.9	(84.2)*	34	770.0	(80.3)	37	723.7	(82.6)
LPA (min/day)	71	173.9	(45.1)	34	169.6	(49.6)	37	177.8	(40.9)
MPA (min/day)	71	94.4	(34.8)	34	94.3	(34.9)	37	94.4	(35.3)
VPA (min/day)	71	1.7	(2.2)*	34	2.3	(2.9)	37	1.1	(1.0)
MVPA (min/day)	71	96.1	(35.4)	34	96.6	(35.5)	37	95.5	(35.8)
Overall physical activity (min/day)	71	269.9	(74.6)	34	266.3	(78.3)	37	273.3	(72.0)
Physical fitness									
VO_2max (ml/min)	71	2339	(657)	34	2915	(373)	37	1810	(332)
VO_2max (ml/kg/min)	71	30.5	(5.6)	34	33.3	(4.5)	37	27.9	(5.3)
Extension peak torque (Nm)	71	266.1	(87.3)	33	334.9	(73.7)	38	202.8	(35.4)
Flexion peak torque (Nm)	71	124.0	(45.6)	33	156.9	(44.3)	38	93.6	(16.6)
Hand grip strength (kg)	73	71.0	(23.7)	35	93.1	(12.1)	38	50.6	(8.2)

Data are presented as means (SD). *Significant differences between sexes (P<0.05)

Table 2. Results of the multiple-regression analysis using the 6 components derived of principal component analysis (i.e. sedentary time and physical activity and physical fitness categories) as predictors S-Klotho.

Component	Multiple R^2	B	β	P
Overall physical activity	0.024	2.707	0.556	0.052
MVPA	0.170	2.764	0.752	0.005
Sedentary time	0.175	0.195	0.046	0.837
VO_2max	0.251	9.132	0.442	0.048
Hand grip strength	0.287	779.399	0.454	0.034
Extension peak torque	0.289	19.440	0.040	0.825
Sex	0.330	273.396	0.385	0.019

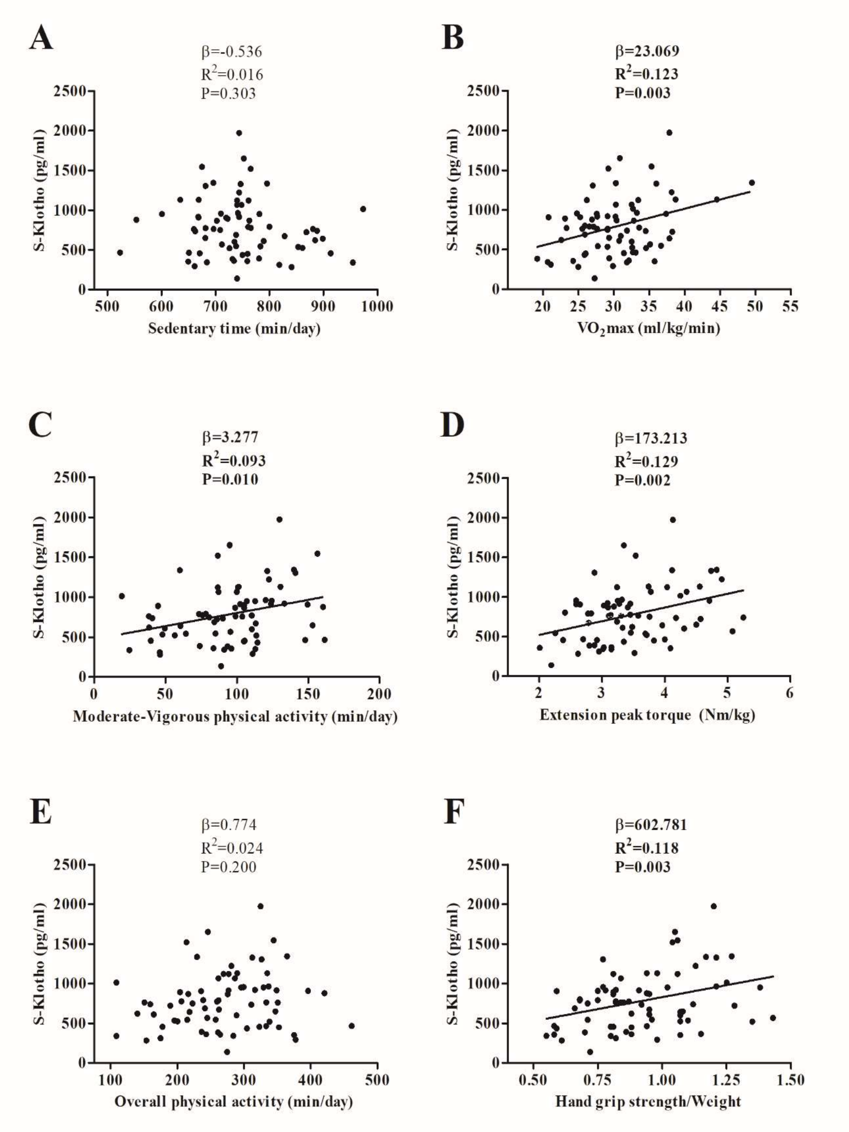

Figure 1. Association between sedentary levels (Figure 1A) maximal oxygen uptake (VO_2max) (Figure 1B), moderate-vigorous physical activity (Figure 1C), extension peak torque (Figure 1D), overall physical activity (Figure 1E), and hand grip strength (Figure 1F) with the S-Klotho in middle-age sedentary adults. β (unstandardized regression coefficient), R^2, and P from a simple linear regression analysis.

VO_2max is considered a powerful predictor of longevity and all-cause mortality in healthy and non-healthy individuals of different ages, regardless of factors such as alcohol, tobacco, or metabolic syndrome [14,19]. Moreover, a positive association between S-Klotho and lower mortality risk was previously reported [31]. However, there is a lack of studies investigating the association between VO_2max and S-Klotho and which physiological mechanism mediates this relationship. Previous studies have described that a single bout of exercise generates cellular oxidative stress [54], while well-designed aerobic exercise programmes (considered a highly effective form to improve VO_2max [14,15]) produce an adaptive response that increases the capacity of cells and organism to withstand greater oxidative stress [55,56]. These studies concluded that there is an inverse relationship between VO_2max and cellular oxidative stress [55,56]. In this sense, S-Klotho increases resistance to cellular oxidative stress through the inhibition of FOXO phosphorylation and of insulin/IGF-1 signalling pathway and upregulating antioxidant enzymes [57–59]. Taken together these findings, it seems plausible that the association of VO_2max with S-Klotho found in our study could be related to cellular oxidative stress, which has been described as a key factor in the ageing process [60].

A recent narrative review suggested that muscular strength is inversely and independently associated with all-cause mortality even after adjusting for cardiorespiratory fitness, age, FM, smoking, or alcohol intake [17]. The relationship between muscular strength and S-Klotho has been previously studied in mice [35] and in older adults (aged>70 years old) [36,37]. Such studies found a positive association, which concurs with the results of the present study conducted in a healthy and relatively younger cohort. These findings could be explained because individuals with higher muscular strength usually have higher levels of LM. As we have recently published in a previous study, the LM seems to be associated with S-Klotho in sedentary middle-aged adults [40]. Some physiological mechanisms could explain this idea. Firstly, previous studies have reported that an impaired α-Klotho gene expression produces a reduction of stem cell frequency [61–63] and an impairment of the angiogenesis process [64] through the Wnt signalling pathways inhibition [62]. Secondly, an alteration of the α-Klotho gene expression generates an increment of TGF-β levels, which promote the mesenchymal transition toward a fibroblastic lineage in the skeletal muscle tissue, among others [61,65]. Considering that the above-mentioned factors are closely related to the age-related sarcopenia (mainly characterised by a loss of skeletal muscle mass), it seems logical that exercise-induced LM increments accompanied by a physical fitness improvement induce positive changes in S-Klotho.

The limitations of the present study include a cross-sectional design, and therefore it is not possible to establish causality. The results of the present study cannot be extrapolated to other populations because the study participants were sedentary middle-aged adults. Finally, due to the relatively small sample size of the current study, the data should be interpreted with caution.

CONCLUSIONS

In summary, our results indicate that physical activity and fitness levels are associated with S-Klotho in sedentary middle-aged adults. Of note is however that these associations are highly dependent on LM. The S-Klotho protein could be a key factor in the relationship between physical activity and physical fitness and health improvements during the ageing process. Further longitudinal studies are needed to elucidate whether changes in physical activity are related to changes in S-Klotho after an exercise training intervention.

REFERENCES

1 López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. *Cell* 2013; **153**.

2 Sen P, Shah PP, Nativio R, Berger SL. Epigenetic mechanisms regulating longevity and aging. *Cell* 2016; **166**: 822–39.

3 Jin K, Simpkins JW, Ji X, Leis M, Stambler I. The Critical Need to Promote Research of Aging and Aging-related Diseases to Improve Health and Longevity of the Elderly Population. *Aging Dis* 2015; **6**: 1.

4 Fulop T, Larbi A, Witkowski JM, McElhaney J, Loeb M, Mitnitski A *et al.* Aging, frailty and age-related diseases. *Biogerontology* 2010; **11**: 547–563.

5 Gruber J, Halliwell B. Approaches for extending human healthspan: from antioxidants to healthspan pharmacology. *Essays Biochem* 2017; **61**: 389–399.

6 Crogan NL. Nutritional Problems Affecting Older Adults. *Nurs Clin North Am* 2017; **52**: 433–445.

7 Rebelo-Marques A, Lages ADS, Andrade R, Ribeiro CF, Mota-Pinto A, Carrilho F *et al.* Aging hallmarks: The benefits of physical exercise. *Front Endocrinol (Lausanne)* 2018; **9**: 1–15.

8 Pedersen B, Saltin B. Exercise as medicine - Evidence for prescribing exercise as therapy in 26 different chronic diseases. *Scand J Med Sci Sport* 2015; **25**: 1–72.

9 Gremeaux V, Gayda M, Lepers R, Sosner P, Juneau M, Nigam A. Exercise and longevity. *Maturitas* 2012; **73**: 312–317.

10 Ekblom-Bak E, Ekblom B, Vikström M, De Faire U, Hellénius ML. The importance of non-exercise physical activity for cardiovascular health and longevity. *Br J Sports Med* 2014; **48**: 233–238.

11 Arem H, Moore SC, Patel A, Hartge P, Berrington De Gonzalez A, Visvanathan K *et al.* Leisure time physical activity and mortality: A detailed pooled analysis of the dose-response relationship. *JAMA Intern Med* 2015; **175**: 959–967.

12 Lee IM, Shiroma EJ, Lobelo F, Puska P, Blair SN, Katzmarzyk PT *et al.* Effect of physical inactivity on major non-communicable diseases worldwide: An analysis of burden of disease and life expectancy. *Lancet* 2012; **380**: 219–229.

13 Patterson R, McNamara E, Tainio M, de Sá TH, Smith AD, Sharp SJ *et al.* Sedentary behaviour and risk of all-cause, cardiovascular and cancer mortality, and incident type 2 diabetes: a systematic review and dose response meta-analysis. *Eur J Epidemiol* 2018; **1**: 1–19.

14 Amaro Gahete FJ, De La O A, Jurado Fasoli L, Castillo MJ, Gutierrez A. Fitness Assessment as an Anti-Aging Marker: A Narrative Review. *J Gerontol Geriatr Res* 2017; **06**.

15 Castillo-Garzón M, Ruiz J, Ortega F,

Gutiérrez A. Anti-aging therapy through fitness enhancement. *Clin Interv Aging* 2006; **1**: 213–220.

16 García-Hermoso A, Cavero-Redondo I, Ramírez-Vélez R, Ruiz JR, Ortega FB, Lee DC *et al.* Muscular Strength as a Predictor of All-Cause Mortality in an Apparently Healthy Population: A Systematic Review and Meta-Analysis of Data From Approximately 2 Million Men and Women. *Arch Phys Med Rehabil* 2018.

17 Volaklis K, Halle M, Meisinger. Muscular strength as a strong predictor of mortality: A narrative review. *Eur J Intern Med* 2015; **26**: 303–310.

18 Lee, M, Artero, EG, Sui, X, Blair S, Lee D, Artero EG, Sui X, Blair SN. Mortality trends in the general population: the importance of cardiorespiratory fitness. *J Psychopharmacol* 2010; **24**: 27–35.

19 Harber M, Kaminsky L, Arena R, Blair S, Franklin B, Myers J *et al.* Impact of cardiorespiratory fitness on all-cause and disease-specific mortality: advances since 2009. *Prog Cardiovasc Dis* 2017.

20 Fitzgerald S, Barlow C, Kampert J, Morrow J, Jackson A, Blair S. Muscular fitness and all-cause mortality: prospective observations. *J Phys Act Heal* 2004; **1**: 7–18.

21 Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T *et al.* Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* 1997; **390**: 45–51.

22 Iida R, Kanko S, Suga T, Morito M, Yamame A. Autophagic-lysosomal pathway functions in the masseter and tongue muscles in the klotho mouse, a mouse model for aging. *Mol Cell Biochem* 2011; **348**: 89–98.

23 Saito Y, Yamagishi T, Nakamura T, Ohyama Y, Aizawa H, Suga T *et al.* Klotho protein protects against endothelial dysfunction. *Biochem Biophys Res Commun* 1998; **248**: 324–9.

24 Kuro-o M. Klotho. *Pflugers Arch Eur J Physiol* 2010; **459**: 333–343.

25 Kim J-H, Hwang K-H, Park K-S, Kong ID, Cha S-K. Biological role of anti-aging protein Klotho. *J lifestyle Med* 2015; **5**: 1–6.

26 Imura A, Iwano A, Tohyama O, Tsuji Y. Secreted Klotho protein in sera and CSF: implication for post-translational cleavage in release of Klotho protein from cell membrane. *FEBS Lett* 2004; **565**: 143–147.

27 Bloch L, Sineshchekova O, Reichenbach D, Reiss K, Saftig P, Kuro-o M *et al.* Klotho is a substrate for alpha-, beta- and gamma-secretase. *FEBS Lett* 2009; **583**: 3221–4.

28 Chang Q, Hoefs S, van der Kemp AW, Topala CN, Bindels RJ, Hoenderop JG. The beta-glucuronidase klotho hydrolyzes and activates the TRPV5 channel. *Science* 2005; **310**: 490–3.

29 Chen C, Podvin S, Gillespie E, Leeman SE, Abraham CR. Insulin stimulates the cleavage and release of the extracellular

domain of Klotho by ADAM10 and ADAM17. *PNAS* 2007; **104**: 19796–19801.

30 Semba R, Cappola A, Sun K. Plasma klotho and cardiovascular disease in adults. *J Am Geriatr Soc* 2011; **59**: 1596–1601.

31 Semba RD, Cappola AR, Sun K, Bandinelli S, Dalal M, Crasto C *et al.* Plasma klotho and mortality risk in older community-dwelling adults. *Journals Gerontol - Ser A Biol Sci Med Sci* 2011; **66 A**: 794–800.

32 Tan S-J, Chu MM, Toussaint ND, Cai MM, Hewitson TD, Holt SG. High-intensity physical exercise increases serum *a* -klotho levels in healthy volunteers. *J Circ Biomarkers* 2018; **7**.

33 Mostafidi E, Moeen A, Nasri H, Ghorbani Hagjo A, Ardalan M. Serum Klotho Levels in Trained Athletes. *Nephrourol Mon* 2016; **8**: e30245.

34 Matsubara T, Miyaki A, Akazawa N. Aerobic exercise training increases plasma Klotho levels and reduces arterial stiffness in postmenopausal women. *Am J Physiol Hear Circ Physiol* 2014; **306**: 348–355.

35 Phelps M, Pettan-Brewer C, Ladiges W, Yablonka-Reuveni Z. Decline in muscle strength and running endurance in klotho deficient C57BL/6 mice. *Biogerontology* 2013; **14**: 729–39.

36 Semba RD, Ferrucci L, Sun K, Simonsick E, Turner R, Miljkovic I *et al.* Low Plasma Klotho Concentrations and Decline of Knee Strength in Older Adults. *J Gerontol A Biol Sci Med Sci* 2016; **71**: 103–8.

37 Semba RRD, Cappola ARA, Sun K, Bandinelli S, Dalal M, Crasto C *et al.* Relationship of low plasma klotho with poor grip strength in older community-dwelling adults: the InCHIANTI study. *Eur J Appl Physiol* 2012; **112**: 1215–20.

38 Belsky DW, Caspi A, Houts R, Cohen HJ, Corcoran DL, Danese A *et al.* Quantification of biological aging in young adults. *Proc Natl Acad Sci* 2015; **112**: E4104–E4110.

39 Fontana L, Kennedy BK, Longo VD. Medical research: Treat ageing. *Nature* 2014; **511**: 405–406.

40 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.

41 Migueles JH, Cadenas-Sanchez C, Ekelund U, Delisle Nyström C, Mora-Gonzalez J, Löf M *et al.* Accelerometer Data Collection and Processing Criteria to Assess Physical Activity and Other Outcomes: A Systematic Review and Practical Considerations. *Sport Med* 2017; **47**: 1821–1845.

42 van Hees VT, Fang Z, Langford J, Assah F, Mohammad A, da Silva ICM *et al.* Autocalibration of accelerometer data for free-living physical activity

assessment using local gravity and temperature: an evaluation on four continents. *J Appl Physiol* 2014; **117**: 738–44.

43 Van Hees VT, Sabia S, Anderson KN, Denton SJ, Oliver J, Catt M *et al.* A novel, open access method to assess sleep duration using a wrist-worn accelerometer. *PLoS One* 2015; **10**: 1–13.

44 Hildebrand M, Hansen BH, van Hees VT, Ekelund U. Evaluation of raw acceleration sedentary thresholds in children and adults. *Scand J Med Sci Sport* 2017; **27**: 1814–1823.

45 Hildebrand M, Van Hees VT, Hansen BH, Ekelund U. Age group comparability of raw accelerometer output from wrist-and hip-worn monitors. *Med Sci Sports Exerc* 2014; **46**: 1816–1824.

46 Balke B, Ware RW. An experimental study of physical fitness of Air Force personnel. *U S Armed Forces Med J* 1959; **10**: 675–88.

47 Borg GA. Psychophysical bases of perceived exertion. *Med Sci Sports Exerc* 1982; **14**: 377–81.

48 Midgley AW, McNaughton LR, Polman R, Marchant D. Criteria for determination of maximal oxygen uptake: A brief critique and recommendations for future research. *Sport Med* 2007; **37**: 1019–1028.

49 Artero, EG, Espada-Fuentes, JC, Argüelles-Cienfuegos, J, Román, A, Gómez-López, PJ, Gutiérrez A. Effects of whole-body vibration and resistance training on knee extensors muscular performance. *Eur J Appl Physiol* 2012; **112**: 1371–1378.

50 Sole G, Hamrén J, Milosavljevic S, Nicholson H, Sullivan SJ. Test-Retest Reliability of Isokinetic Knee Extension and Flexion. *Arch Phys Med Rehabil* 2007; **88**: 626–631.

51 Ruiz-Ruiz J, Mesa J, Gutiérrez A, Castillo M. Hand size influences optimal grip span in women but not in men. *J Hand Surg Am* 2002; **27**: 897–901.

52 Crasto C, Semba R, Sun K, Cappola A, Bandinelli S, Ferrucci L. Relationship of low-circulating "anti-aging" klotho hormone with disability in activities of daily living among older community-dwelling adults. *Rejuvenation Res* 2012; **5**: 295–301.

53 Amaro-Gahete FJ, de la O A, Jurado-Fasoli L, Ruiz JR, Castillo MJ, Gutierrez A. Role of exercise on S-Klotho protein regulation: a systematic review. *Curr Aging Sci* 2018; **11**.

54 Powers SK, Radak Z, Ji LL. Exercise-induced oxidative stress: past, present and future. *J Physiol* 2016; **594**: 5081–5092.

55 Traustadóttir T, Davies SS, Su Y, Choi L, Brown-Borg HM, Roberts LJ *et al.* Oxidative stress in older adults: Effects of physical fitness. *Age (Omaha)* 2012; **34**: 969–982.

56 Rosado-Pérez J, Mendoza-Núñez VM. Relationship Between Aerobic Capacity

With Oxidative Stress and Inflammation Biomarkers in the Blood of Older Mexican Urban-Dwelling Population. *Dose-Response* 2018; **16**.

57 Zeldich E, Chen C, Colvin TA, Bove-fenderson EA, Liang J, Zhou TBT *et al.* The Neuroprotective Effect of Klotho is Mediated via Regulation of Members of the Redox System. *J Biol Chem* 2014; **289**: 24700–24715.

58 Yamamoto M, Clark JJD, Pastor J V., Gurnani P, Nandi A, Kurosu H *et al.* Regulation of oxidative stress by the anti-aging hormone klotho. *Biol Chem* 2005; **280**: 3829–3824.

59 Kuro-o M. Klotho as a regulator of oxidative stress and senescence. *Biol Chem* 2008; **389**: 233–241.

60 Bouzid MA, Filaire E, McCall A, Fabre C. Radical oxygen species, exercise and aging: an update. *Sport Med* 2015; **45**: 1245–1261.

61 Avin KG, Coen PM, Huang W, Stolz DB, Sowa GA, Dubé JJ *et al.* Skeletal muscle as a regulator of the longevity protein, Klotho. *Front Physiol* 2014; **5**: 189.

62 Liu H, Fergusson MM, Castilho RM, Liu J, Cao L, Chen J *et al.* Augmented Wnt signaling in a mammalian model of accelerated aging. *Science* 2007; **317**: 803–6.

63 Izbeki F, Asuzu DT, Lorincz A, Bardsley MR, Popko LN, Choi KM *et al.* Loss of Kit low progenitors , reduced stem cell factor and high oxidative stress underlie gastric dysfunction in progeric mice. 2010; **16**: 3101–3117.

64 Fukino K, Suzuki T, Saito Y, Shindo T, Amaki T, Kurabayashi M *et al.* Regulation of angiogenesis by the aging suppressor gene klotho. 2002; **293**: 332–337.

65 Doi S, Zou Y, Togao O, Pastor J, John G, Wang L. Klotho inhibits transforming growth factor-beta1 (TGF-beta1) signaling and suppresses renal fibrosis and cancer metastasis in mice. *J Biol Chem* 2011; **286**: 8655–65.

SECTION 2: S-Klotho protein, energy metabolism and cardiometabolic health

This section includes two chapters aiming to study the relationship of energy metabolism, cardiometabolic health and S-Klotho in sedentary middle-aged adults. It has been analized: [i] the association of BMR and fuel oxidation in basal conditions and during exercise with S-Klotho (**Study 11**), and [ii] the association of S-Klotho with cardiometabolic risk with in sedentary middle-aged adults (**Study 12**).

Chapter 7:
Association of basal metabolic rate and fuel oxidation in basal conditions and during exercise, with S-Klotho
(Study 11)

ABSTRACT

S-Klotho, the shed form of a-klotho, is thought to be an ageing suppressor with functions related to the physiology of energy metabolism. However, it remains unknown whether BMR and fuel oxidation in basal conditions and during exercise are associated in any way with ageing biomarkers such as S-Klotho and/or chronological ageing. The present work investigates the association of BMR and fuel oxidation in basal conditions and during exercise, with S-Klotho in middle-aged, sedentary adults.

BMR was measured by indirect calorimetry in 74 such subjects (53% women; age 53.7±5.1 years) following standard procedures, and their fuel oxidation estimated via stoichiometric equations. MFO and Fat_{max} *were determined using a walking graded exercise test.*

No relationship was seen between BMR and S-Klotho (P>0.1), although both basal fat oxidation and MFO showed positive associations with this protein (both P<0.001); these relationships persisted after controlling for age, sex and FM. However, no significant associations were seen between BMR, BFox or MFO and chronological age (all P>0.1).

The present findings suggest that BFox and MFO are strongly associated with S-Klotho in middle-aged sedentary adults. These results support the idea that metabolic flexibility is a powerful predictor of biological ageing.

BACKGROUND

Life expectancy in Europe has generally increased in recent decades. In 2012, 17% of the European Union population was aged 65 years or older, a percentage expected to rise to 25% by 2035, and to 30% in 2060 [1]. However, a longer life expectancy does not necessarily mean healthy ageing; it can mean extra years of suffering chronic disease, particularly metabolic illnesses such us obesity and diabetes mellitus type II [1,2].

Ageing is characterized by a progressive decline in one's metabolic and physiological functions [3], the associated dysregulation of nutrient sensitivity, mitochondrial dysfunction and cellular apoptosis eventually becoming harmful [4] Ageing is associated with a progressive decline in the BMR, meal-induced thermogenesis and physical activity [5], resulting in a reduced total energy expenditure. In part, this is responsible for the gradual weight increase and the deposition of VAT seen during ageing, which places people at greater risk of cardiometabolic disease and all-cause mortality [6].

Over the last decade, numerous studies have examined the association between basal fuel oxidation and ageing-related diseases, and a potential role for this oxidation has been proposed in the pathogenesis of subclinical atherosclerosis, hypertriglyceridaemia, liver steatosis and ventricular cardiac remodelling [7–9]. Ageing is positively associated with visceral adiposity, but in a study involving a large and heterogeneous adult population, no relationship was observed between basal substrate oxidation and chronological age [3]. Recent studies have suggested that chronological age is but a crude indicator of ageing. Specific ageing biomarkers provide a more accurate picture; indeed, they provide a reliable tool for understanding and assessing ageing [10].

The α-klotho gene is thought to suppress ageing, extending life expectancy when it is overexpressed and inducing premature ageing when it is defective [11,12]. It is mainly expressed in the kidney, the parathyroid glands and the brain; its product is a type-1 single-pass transmembrane glycoprotein, the ectodomain of which is shed and released into the systemic circulation in soluble form [13]. S-Klotho has several functions related to the physiology of energy metabolism [14], including the regulation of glucose uptake, the enhancement of insulin sensitivity, the attenuation of cellular oxidative stress, and the suppression of chronic inflammation [15–17], which together are thought to invest it with anti-ageing properties. A recent study showed that S-Klotho is lower in individuals with diabetes mellitus type II, and therefore a potential biomarker of this disease [18]. It thus seems plausible that individuals with a reduced BMR and an altered fuel oxidation in basal conditions and during exercise may have lower S-Klotho. The literature contains no studies on how BMR and fuel oxidation in basal conditions and during exercise may be related to chronological ageing, or whether they have any relationship with ageing biomarkers such as S-Klotho. The aim of the present work was to investigate the relationship of BMR and fuel oxidation in basal conditions and during exercise, with S-Klotho.

MATERIAL & METHODS

Study design and participants

This cross-sectional study was performed as part of the FIT-AGEING project (clinicaltrial.gov: ID: NCT03334357) [19]. Eighty-nine middle-aged, sedentary adults were initially recruited, of whom 15 were excluded from analysis due to problems in data collection or usage; the final number of study subjects was therefore 74 (~52% women). Subjects were recruited through advertisements distributed in the form of leaflets and via social networks and electronic media. The inclusion criteria were: (i) age 45-65 years old, (ii) practicing <20 min of physical activity on <3 days per week (self-reported), (iii) to be taking no drug or long-term medication, (iv) to be a non-smoker, (v) to have no cardiometabolic illness, (vi) to not be pregnant, (vii) and to have experienced no significant weight change (<3 kg) in the past 12 weeks.

All subjects gave their written, informed consent to be included in accordance with the latest revision of the Declaration of Helsinki (2013). The study was approved by the Human Research Ethics Committee of the *Junta de Andalucía* [0838-N-2017].

Procedures

All assessments were made at the *Centro de Investigación Deporte y Salud (CIDS)* during September and October of 2016 and 2017. Subject weight and height were measured using a Seca model 799 scale and stadiometer (Seca, Hamburg, Germany), and the BMI calculated as (*weight [kg]/ height² [m]*). FM, VAT mass and LM were determined using a Discovery Wi dual-energy X-ray absorptiometer (Hologic, Inc., Bedford, MA, USA). The FMI and the LMI were calculated as *(FM [kg]/ height² [m])* and *([kg]/ height2 [m])* respectively.

Subjects were told to arrive at the laboratory in a motor vehicle, and to avoid any moderate/vigorous physical activity in the previous 24 h/48 h respectively; all were required to confirm that they had met this condition. BMR was determined by IC in a peaceful room at 22-24ºC and 35-45% humidity, at between 8 and 10 a.m. following a 12 h fast, using an Ultima CardiO2 metabolic cart (Medgraphics Corp, MN, USA) and employing a neoprene face-mask with no external ventilation [20]. The evening meal consumed by subjects prior to fasting was standardized: an egg omelette with fried tomato and boiled rice. The Ultima CardiO2 metabolic cart device assessed VO_2 using a galvanic fuel cell, and VCO_2 via non-dispersive infrared analysis using a breath-by-breath system [21]. Prior to the start of BMR assessment, the subjects reclined on a bed for ~30 min in a comfortable supine position, covered by a sheet [22,23]. Meanwhile, a gas calibration using two standard gas concentrations, and a flow calibration using a 3 L calibration syringe, were performed following the manufacturer's instructions. BMR and basal fuel oxidation were measured over a 30 min period in which the participants were instructed to breath normally, neither talking, fidgeting nor sleeping. The first 5 min of each dataset were discarded. CV for VO_2, VCO_2, the RER, and minute ventilation, were calculated for 5 min intervals (i.e., from the 1st to the 5th min, from 2nd to 6th, from 3rd to 7th, etc). In accordance with previous studies [24,25], the 5 min periods that met steady-state gas exchange criteria (i.e., CV<10% in VO_2, CO_2, and minute ventilation, and CV<10% in RER) were then selected, and the 5 min period with the lowest CV for VO_2, VCO_2, RER, and minute ventilation chosen for further analysis (excluding those subjects with a RER of <0.7 or >1.0). Weir's abbreviated equation [26] was used to estimate the BMR, and Frayn equations [27] were used to estimate BFox and BCHox expressed in g/min. The BMR was also calculated with respect to the LM (BMR_{LM}). The BFox and BCHox were also expressed as a percentage of the BMR.

MFO and Fat_{max}, were determined via a walking graded exercise test on a H/P/Cosmos Pulsar treadmill (H/P/Cosmos Sports & Medical GmbH, Nussdorf-Traunstein, Germany). The maximum walking speed was assessed following the methodology used in previous studies [28–30]. The walking graded exercise test started with a warm-up at 3.5 km/h and a 0% gradient, and the speed then increased by 1 km/h every 3 min until the maximum walking speed was reached. The gradient was then increased by 2% every 3 min until the RER was >1.0. The subjects wore a Model 7400 face mask (Hans Rudolph Inc, Kansas City, MO, USA) equipped with a prevent™ metabolic flow sensor (Medgraphics Corp, Minnesota, USA) connected to the Ultima CardiO_2 metabolic cart for measuring gas exchange. Gas and flow calibrations were performed following the manufacturer's instructions. VO_2 and VCO_2 data were averaged every 10 s using Breeze Suite software v.8.1.0.54. Fat oxidation was calculated from the RER during the last 60 s of each stage in the graded exercise test, using standard IC equations [27]. As previously described, MFO and $Fatm_{ax}$ were estimated via a 3rd polynomial curve with fat oxidation as a function of VO_2max [30]. The MFO was also determined with respect to LM (MFO_{LM}).

Following the modified Balke protocol [31], a maximal graded exercise test was used to determine VO_2max on another day (interval 3-7 days). Subjects were asked: (i) to fast for 3 to 5 h, but eating a complete meal just before, (ii) to avoid drugs and/or stimulants at least 24 h before the test, and (iii) to refrain from moderate and/or vigorous physical activity for 24 h/48 h before the test respectively. Briefly, the test began at a speed of 3.5 km/h (gradient 0%), increasing until reaching 5.3 km/h. The gradient was then increased by 1% every minute, keeping the treadmill speed constant until subject exhaustion. The heart rate was continuously monitored and recorded every 5 s using a Polar RS800 heart rate monitor (Polar Electro Oy, Kempele, Finland).

Blood samples were obtained from the antecubital vein in the morning just before BMR assessment. S-Klotho was determined in EDTA plasma using a solid-phase sandwich ELISA kit (Demeditec, Kiel, Germany). Optical density was assessed at 450 nm ± 2 nm. The intra- and inter-assay CV (3-10% each) was determined using two different doses of pure S-Klotho.

Statistical analysis

The normal distribution of all variables was confirmed using the Shapiro-Wilk test, visual histograms, Q-Q plots and box plots. The Student t test for unpaired samples was used to examine differences in the results of male and female subjects. Given the aim of the study, and the lack of any significant interaction between sex (all P>0.05), the appropriateness of fitting models for men and women were combined including sex as a covariable.

Simple linear regression models were first used to examine the association of BMR, BMR_{LM}, BFox, BCHox, MFO, MFO_{LM}, and Fat_{max} with S-Klotho. Multiple linear regression analyses were then conducted to study these associations while controlling for potential confounders: (i) Model 1 was adjusted for age; (ii) Model 2 for sex; and (iii) Model 3 for FM percentage. These potential confounders were selected on the basis of theoretical considerations and the results of stepwise regression. Simple linear regression was also performed to examine the association of BMR, BMR_{LM}, BFox, BCHox, MFO, MFO_{LM} and Fat_{max} with chronological age. All calculations were made using the Statistical Package for the Social Sciences v.22.0 (IBM Corporation, Chicago, IL, USA). GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA) was used for graphical plots. Significance was set at P≤0.05.

RESULTS

Table 1 summarises the descriptive characteristics of the study subjects. BMR and BMR_{LM} showed no significant association with S-Klotho (Figure 1A and 1B; P>0.1), a result that persisted after controlling for age, sex and FM (Table 2; P>0.05). A significant, negative association was detected between BCHox (expressed in g/min, and in % BMR) and S-Klotho (Figure 1E and 1F; all P≤0.001), while a significant positive association was seen between BFox (expressed in g/min, and in % BMR) and S-Klotho (Figure 1C, and 1D; all P<0.001). These associations persisted after controlling for age, sex, and FM (Table 2; P≤0.01).

MFO was significantly associated with S-Klotho (Figure 2A; P=0.034, β=1104.7) even after controlling for age, sex, and FM (Table 2; P≤0.04). Neither MFO_{LM} nor Fat_{max} showed any relationship with S-Klotho (Figure 2B and 2C; all P>0.1), a finding that persisted after adjusting for age, sex, and FM (Table 2; P>0.08).

Neither BMR, BMR_{LM}, BFox, BCHox, MFO, MFO_{LM} nor Fat_{max} showed an association with chronological age (Figure 3 and Figure 4; all P>0.1); these findings persisted after adjusting for sex and FM (data not shown).

All of the above-mentioned analyses were also run adjusting for VAT, VO_2max, objectively measured moderate-vigorous physical activity, and total energy intake, and all findings persisted (data not shown).

DISCUSSION

The main findings of the present study are that the capacity to oxidase fat in basal conditions and during exercise (i.e., MFO), are positively associated with S-Klotho, while BCHox is inversely associated with the latter, in sedentary middle-aged adults. Neither BMR nor fuel oxidation showed any association with chronological age, either in basal conditions or during exercise.

BMR accounts for ~70% of total energy expenditure, and is largely responsible for overall energy homeostasis [32]. BMR falls by 1-2% per decade after 20 years of age, and is closely linked to the progressive reduction in LM seen with ageing [33]. Our group recently reported a strong association between LM and S-Klotho [34] but, paradoxically, no association was seen between BMR and S-Klotho in the present work. This might be explained in that the age of the present subjects was quite homogeneous, and because factors (in addition to the LM) such us energy flux rates, mitochondrial proton leakage, protein turnover, and Na+–K+–ATPase activity can influence the BMR during ageing [35]. Future studies with larger sample sizes and with a wide range of subject ages are needed to confirm these findings, and to examine whether changes in BMR are associated with changes in S-Klotho.

Metabolic flexibility, defined as the ability to increase fat oxidation upon increased fatty acid availability, and/or to switch between fat and carbohydrate oxidation as the primary fuel source [36], undergoes important changes during the ageing process [33].

Ageing is characterized by a progressive qualitative and quantitative decline in LM, poor mitochondrial volume and efficiency, a reduction in type II muscle fibre size, lower capillary density, resistance to anabolic endocrine signals, and a more pro-inflammatory environment [4]. Together, these changes underlie the theoretical framework for the appearance of metabolic inflexibility with ageing. Conflicting results have been reported over time regarding the relationship between basal fuel oxidation and ageing. Initially, some studies reported a reduced BFox in older individuals compared to their younger counterparts [37,38].

Table 1. Study participant characteristics.

	N	All		N	Men		N	Women	
Age (years)	74	53.7	(5.1)	35	54.4	(5.3)	39	53.0	(5.0)
S-Klotho (pg/ml)	73	775.3	(363.7)	34	814.1	(452.2)	39	741.4	(265.6)
Anthropometry and body composition									
Weight (kg)	74	75.7	(15.0)	35	87.4	(11.0)	39	65.3	(9.3)*
Height (cm)	74	167.8	(9.8)	35	175.8	(6.5)	39	160.7	(6.1)*
BMI (kg/m^2)	74	26.7	(3.8)	35	28.3	(3.6)	39	25.3	(3.3)*
FM (kg)	74	30.0	(8.4)	35	30.9	(9.8)	39	29.2	(7.1)
FM (%)	74	39.9	(9.1)	35	34.7	(8.0)	39	44.5	(7.4)*
FMI (kg/m^2)	74	10.7	(3.1)	35	10.0	(3.2)	39	11.4	(2.9)
VAT (g)	74	789.7	(387.1)	35	972.4	(392.0)	39	625.8	(303.4)*
LM (kg)	74	43.5	(11.7)	35	53.9	(6.5)	39	34.1	(5.8)*
LMI (kg/m^2)	74	15.2	(2.9)	35	17.5	(20.0)	39	13.2	(1.8)*
BMR and fuel oxidation under post-fast baseline conditions									
BMR (kcal/day)	71	1508.4	(364.5)	34	1805.5	(244.8)	37	1235.5	(208.4)*
BMR_{LM} ($kcal/kg_{leanmass}/day$)	71	35.2	(7.2)	34	33.6	(5.3)	37	36.7	(8.4)
BFox (g/min)	71	0.053	(0.040)	34	0.064	(0.050)	37	0.042	(0.025)*
BFox (% BMR)	71	45.6	(30.0)	34	45.6	(32.7)	37	45.6	(27.7)
BCHox (g/min)	71	0.112	(0.096)	34	0.138	(0.115)	37	0.089	(0.069)*
BCHox (% BMR)	71	41.8	(32.0)	34	44.0	(34.7)	37	39.8	(29.0)
Fuel oxidation during exercise									
MFO (g/min)	71	0.29	(0.09)	34	0.35	(0.09)	37	0.23	(0.04)*
MFO_{LM} ($g/kg_{leanmass}/min$)	71	6.72	(1.61)	34	6.43	(1.49)	37	6.99	(1.70)
Fat_{max} (%VO_2max)	71	43.0	(10.4)	34	41.6	(10.3)	37	44.3	(10.6)
Cardiorespiratory fitness									
VO_2max (ml/min)	71	2339.2	(657.2)	34	2915.4	(373.2)	37	1809.7	(332.5)*
VO_2max (ml/kg/min)	71	30.5	(5.6)	34	33.3	(4.5)	37	27.9	(5.3)*

Data are presented as means (SD). *Significant differences between sexes obtained via the T-Student unpaired-samples test (P<0.05). Abbreviations: BMR; Basal Metabolic Rate, BMRLM; Basal Metabolic Rate relative to Lean Mass, BFox; Basal Fat Oxidation, BCHox; Basal Carbohydrate Oxidation, MFO; Maximal Fat Oxidation during exercise, MFO_{LM}; MFO relative to Lean Mass, Fat_{max}; Intensity of exercise that elicits MFO, VO_2max; Maximal Oxygen Uptake.

Table 2. Association of basal metabolic rate, basal fat oxidation, basal carbohydrate oxidation, maximal fat oxidation (MFO) and the intensity of exercise that elicits maximal fat oxidation (Fat_{max}) with S-Klotho, adjusted for age (Model 1), sex (Model 2), and FM (Model 3).

	S-Klotho					
	Model 1		Model 2		Model 3	
	P value	β	P value	β	P value	β
BMR (kcal/day)	0.209	0.184	0.203	0.247	0.670	0.051
BMR_{LM} ($kcal/ kg_{leanmass}/day$)	0.150	10.495	0.091	15.868	0.490	9.180
BFox (g/min)	**<0.001**	**3340.712**	**<0.001**	**5380.689**	**<0.001**	**4701.526**
BFox (% BMR)	**0.001**	**3.536**	**<0.001**	**6.434**	**<0.001**	**5.895**
BCHox (g/min)	**0.010**	**-887.501**	**<0.001**	**-2038.484**	**<0.001**	**-1723.932**
BCHox (% BMR)	**0.002**	**-3.204**	**<0.001**	**-6.145**	**<0.001**	**-5.580**
MFO (g/min)	**<0.001**	**1312.915**	**0.036**	**1429.228**	**0.009**	**715.126**
MFO_{LM} ($g/kg_{leanmass}/min$)	0.956	1.120	0.294	-29.664	0.891	3.964
Fat_{max} (% of VO_2max)	0.724	1.108	0.133	-6.349	0.078	-6.886

P value of multiple-regression analysis. β (unstandardized regression coefficient). Abbreviations: BMR; Basal Metabolic Rate, BMR_{LM}; Basal Metabolic Rate relative to lean mass, BFox; Basal Fat Oxidation, BCHox; Basal Carbohydrate Oxidation, MFO_{LM}; MFO relative to lean mass, Fat_{max}; Intensity of exercise that elicits MFO, VO_2max; Maximal Oxygen Uptake.

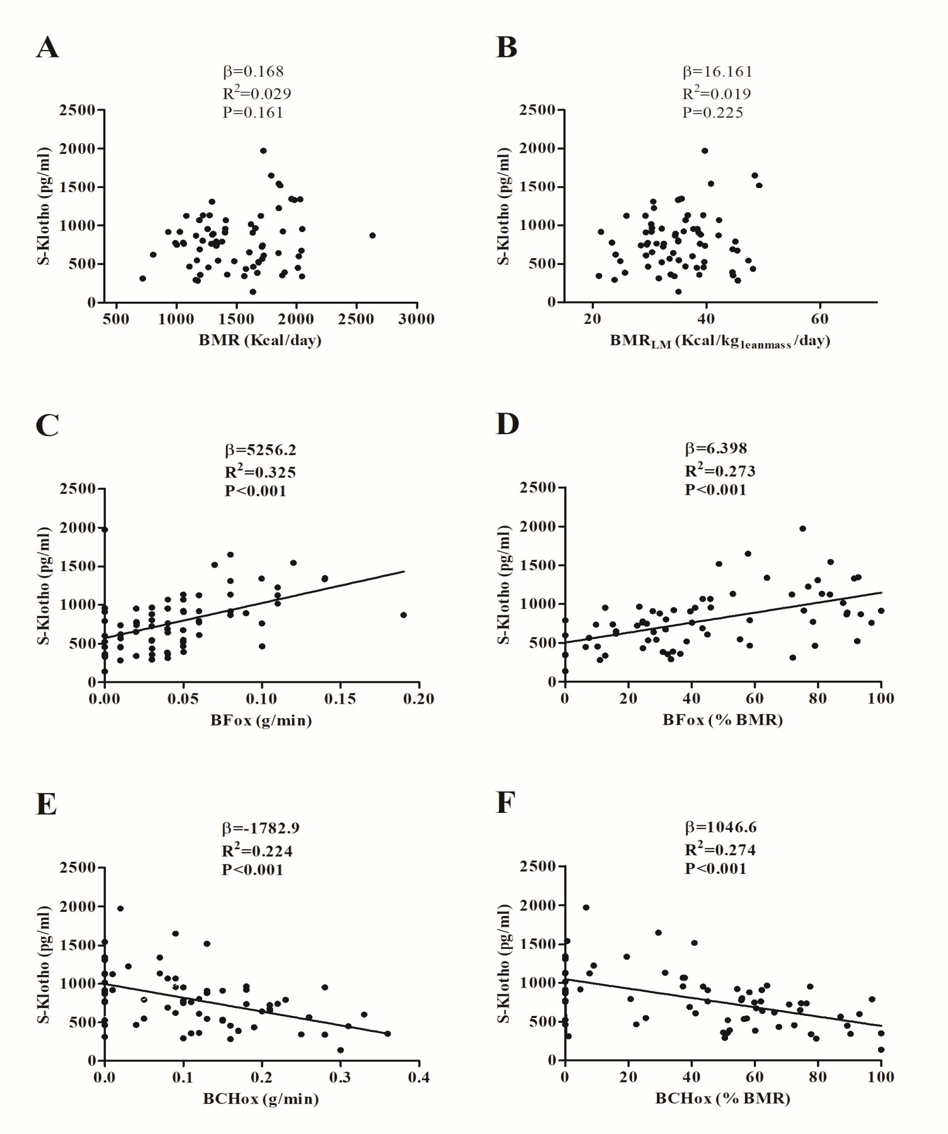

Figure 1. Association between basal metabolic rate (BMR, Figure 1A and 1B), basal fat oxidation (BFox, Figure 1C and 1D) and basal carbohydrate oxidation (BCHox, Figure 1E and 1F) with S-Klotho. β (unstandardized regression coefficient), R^2, and P are from simple linear regression analysis. Abbreviations: BMR_{LM}; Basal Metabolic Rate relative to lean mass.

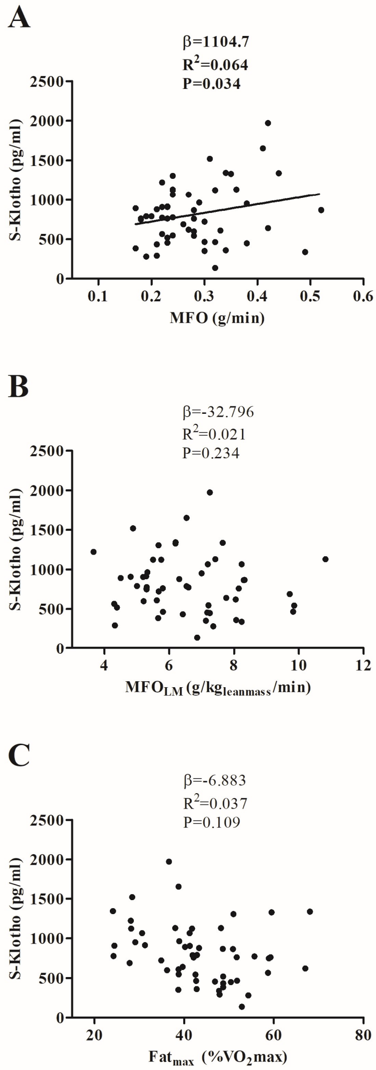

Figure 2. Association between maximal fat oxidation (MFO, Figure 2A and 2B), and the intensity of exercise that elicits MFO (Fat_{max}, Figure 2C) with S-Klotho. β (unstandardized regression coefficient), R^2 and P are from simple linear regression analysis. Abbreviations: MFO; Maximal Fat Oxidation, MFO_{LM}; Maximal Fat Oxidation relative to lean mass, Fat_{max}; Intensity of exercise that elicits MFO, VO_2max; Maximal Oxygen Uptake.

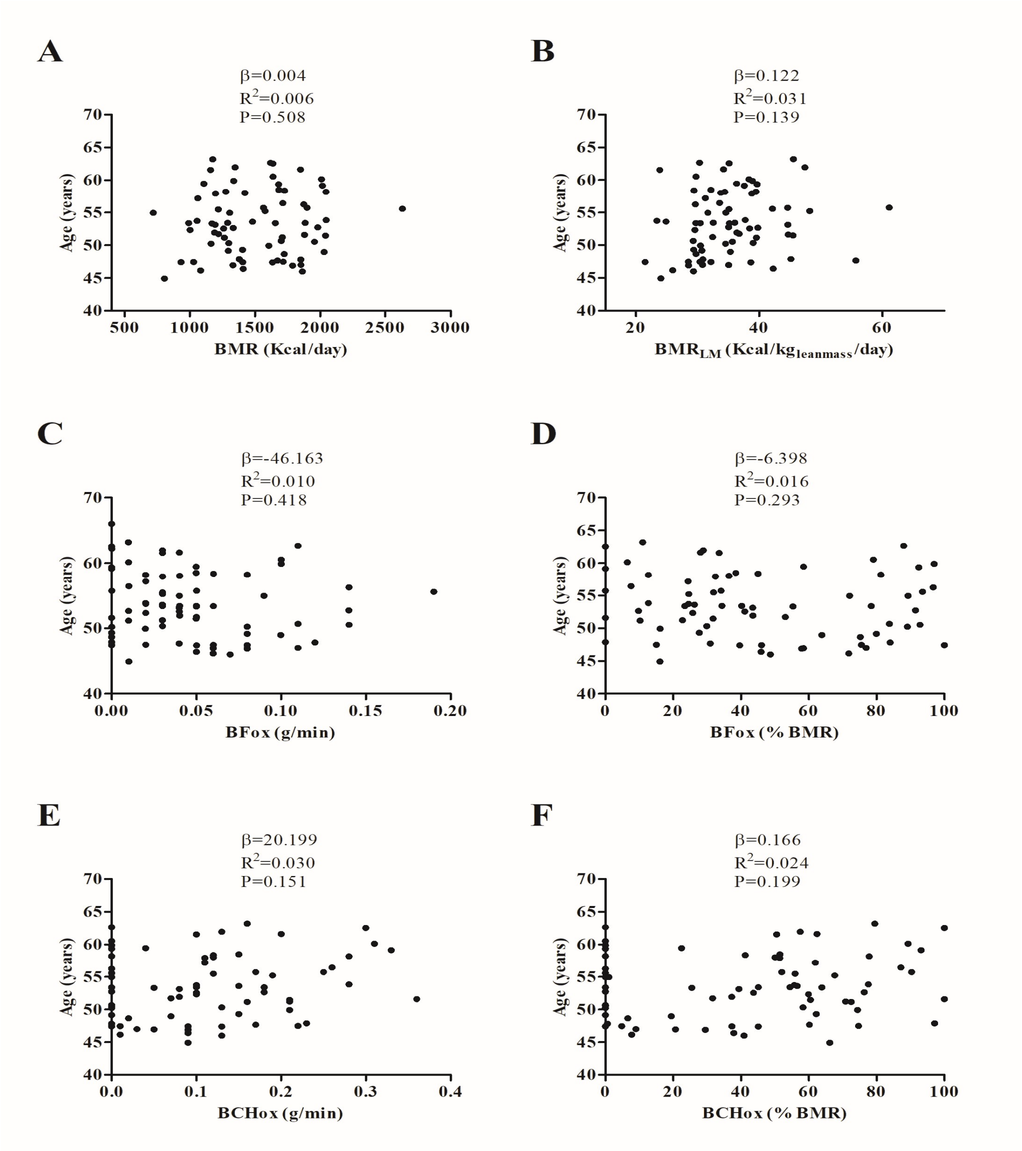

Figure 3. Association between basal metabolic rate (BMR, Figure 1A and 1B), basal fat oxidation (BFox, Figure 1C and 1D) and carbohydrate oxidation (BCHox, Figure 1E and 1F) with age. β (unstandardized regression coefficient), R^2 and P are from a simple linear regression analysis. Abbreviations: BMR_{LM}; Basal Metabolic Rate relative to lean mass.

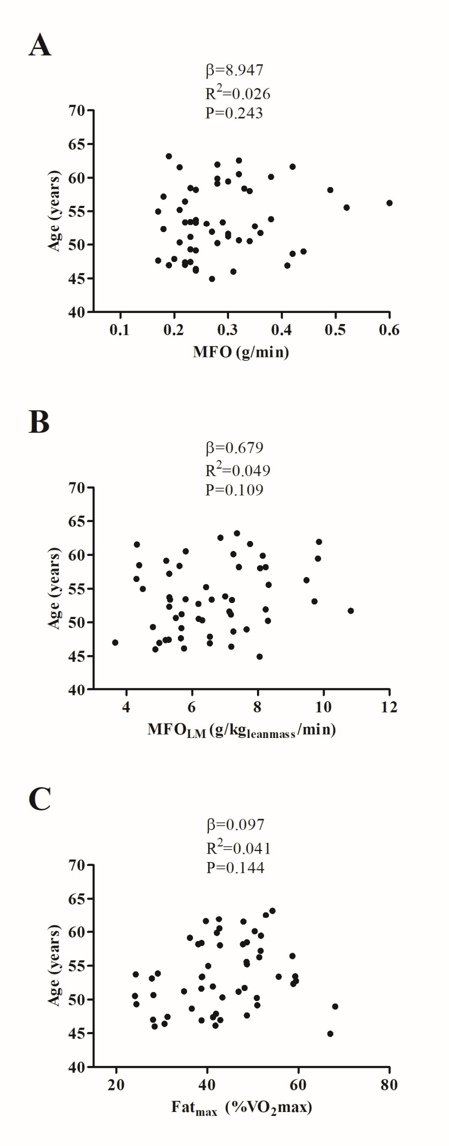

Figure 4. Association between maximal fat oxidation (MFO, Figure 2A and 2B), and the intensity of exercise that elicits MFO (Fat_{max}, Figure 2C) with S-Klotho. β (unstandardized regression coefficient), R^2 and P are from a simple linear regression analysis. Abbreviations: MFO; Maximal Fat Oxidation, MFO_{LM}; Maximal Fat Oxidation relative to lean mass, Fat_{max}; Intensity of exercise that elicits MFO, VO_2max; Maximal Oxygen Uptake.

However, methodological issues may have influenced these findings (e.g., small sample sizes, narrow and limiting inclusion criteria, poorly defined age groups [young vs. old], different data collection methods, and the method of determining fuel oxidation, etc.). In response, Siervo et al. [3], recently conducted an elegant study to examine the association between BFox and chronological age in a large cohort (3442 individuals [2465 women] aged 18-81 years), using a ventilated-hood IC system to determine fuel oxidation. In agreement with the present findings, but contrary to their own hypothesis, these authors found no significant association between BFox and chronological ageing [3]. They suggested this lack of association might be explained by age-related changes in metabolic flexibility becoming more evident when the fuel oxidation capacity becomes crucial in the regulation of metabolic homeostasis (i.e., in the post-prandial state) [3]. Although ageing has typically been understood in terms of chronological age, several studies have suggested that it is a crude yardstick given the heterogeneity in individuals' physiology and health-related outcomes; further, the influence of ageing is different between individuals, and even at the organ/tissue level of the same individual [10]. Measuring biological ageing biomarkers might therefore provide a more valid and reliable tool for assessing and examining the ageing process [10].

S-Klotho is understood to be a powerful anti-ageing biomarker. It functions as a human ageing-suppression molecule and has pleiotropic activities that result in the protection of tissues and organ [39,40] Indeed, previous studies have reported a positive relationship between S-Klotho and life span [41], and an inverse association with coronary artery disease , atherosclerosis [42], osteoporosis [43], calcinosis, stroke [44], acute and chronic kidney diseases [45], different cancers [46], salt-sensitive hypertension [47] and all-cause mortality [47]. The transmembrane klotho protein is an essential component of endocrine FGFR complexes, which have a key role in the pathophysiology of ageing-related disorders via the mediation of phosphate and calcium homeostasis [40]. However, S-Klotho cannot function as a soluble receptor of FGF, and a number of FGF-independent functions have been described for it in the homeostasis of energy metabolism [14,15,40]. The anti-ageing properties of S-Klotho have been thought partially owed to its specific metabolic function: 1) It inhibits insulin and IGF-1)receptors, preventing their phosphorylation by the modification of their glycans [12]. Insulin induces transmembrane klotho shedding, and the consequent increase in S-Klotho inhibits insulin signalling in peripheral tissues and impedes the prolonged action of insulin [15,40]. This partial inhibition of insulin and IGF-1 is an evolutionarily conserved mechanism for suppressing ageing via the enhancement of insulin sensitivity [15,40]. 2) After binding to different Wnt ligands, it inhibits Wnt signalling and promotes stem cell proliferation and survival [48]. 3) It increases resistance to oxidative stress by inhibiting FOXO phosphorylation and upregulating a number of antioxidant enzymes [49,50].

Recent studies have shown that S-Klotho production is downregulated in persons with diabetes mellitus type II; such patients experience hyperglycaemia, insulin resistance and an attenuated resistance to oxidative stress [18]. The reduced presence of S-Klotho in these individuals who are metabolically inflexible in response to different stressors [36] hints at metabolic flexibility and S-Klotho levels being closely associated - and the present work shows a strong association between metabolic flexibility (both under post-fast baseline conditions and during exercise) with S-Klotho.

Limitations

The present work suffers from a number of limitations. Given its cross-sectional design, no causal interpretation can be established; the sample size in relatively small; and only sedentary adults aged 45-65 years were included. These findings may not be extrapolatable to older, younger, and/or trained individuals.

CONCLUSIONS

In summary, the present results suggest that BFox and MFO are strongly associated with the S-Klotho concentration in middle-aged, sedentary adults. However, no relationship was observed between BFox and MFO with chronological age under either set of test conditions. These results have clinical implications, and support the idea that BFox and MFO are powerful predictors of biological ageing. Further studies are needed to examine whether metabolic flexibility in response to other stressors (i.e., the post-prandial state, or after cold exposure, etc.) are associated with S-Klotho. A longitudinal intervention aiming to improve fat oxidation should be performed to determine whether S-Klotho increases in parallel.

REFERENCES

1 Mathus-Vliegen EMH. Obesity and the elderly. *J Clin Gastroenterol* 2012; **46**: 533–544.

2 Rössner S. Obesity in the elderly – a future matter of concern ? *Obes Rev* 2001; : 183–188.

3 Siervo M, Lara J, Celis-Morales C, Vacca M, Oggioni C, Battezzati A *et al.* Age-related changes in basal substrate oxidation and visceral adiposity and their association with metabolic syndrome. *Eur J Nutr* 2016; **55**: 1755–67.

4 López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. *Cell* 2013; **153**.

5 St-Onge M-P, Gallagher D. Body composition changes with aging : The cause or the result of alterations in metabolic rate and macronutrient oxidation ? *Nutrition* 2010; **26**: 152–155.

6 Tchernof A, Després J. Pathophysiology of human visceral obesity: an update aspects of regional body fat distribution. *Physiol Rep* 2013; **93**: 359–404.

7 Montalcini T, Lamprinoudi T, Gorgone G, Ferro Y, Romeo S, Pujia A. Subclinical cardiovascular damage and fat utilization in overweight/obese individuals receiving the same dietary and pharmacological interventions. *Nutrients* 2014; **6**: 5560–71.

8 Montalcini T, Lamprinoudi T, Morrone A, Mazza E, Gazzaruso C, Romeo S *et al.* Nutrients utilization in obese individuals with and without hypertriglyceridemia. *Nutrients* 2014; **6**: 790–8.

9 Montalcini T, Gazzaruso C, Ferro Y, Migliaccio V, Rotundo S, Castagna A *et al.* Metabolic fuel utilization and subclinical atherosclerosis in overweight/obese subjects. *Endocrine* 2013; **44**: 380–5.

10 Foo H, Mather KA, Thalamuthu A, Sachdev PS. The many ages of man: diverse approaches to assessing ageing-related biological and psychological measures and their relationship to chronological age. *Curr Opin Psychiatry* 2019; **32**: 130–137.

11 Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T *et al.* Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* 1997; **390**: 45–51.

12 Kurosu H, Yamamoto M, Clark J, Pastor J, Nandi A, Gurnani P *et al.* Suppression of aging in mice by the hormone Klotho. *Science (80-)* 2005; **309**: 1829–33.

13 Imura A, Iwano A, Tohyama O, Tsuji Y. Secreted Klotho protein in sera and CSF: implication for post-translational cleavage in release of Klotho protein from cell membrane. *FEBS Lett* 2004; **565**: 143–147.

14 Razzaque MS. The role of Klotho in energy metabolism. *Nat Rev Endocrinol* 2012; **8**: 579–587.

15 Kuro-o M. Klotho. *Pflugers Arch Eur J Physiol* 2010; **459**: 333–343.

16 Kuro-o M. Molecular Mechanisms Underlying Accelerated Aging by Defects in the FGF23-Klotho System. *Int J Nephrol*

2018; **2018**: 1–6.

17 Kuro-o M. Klotho and the Aging Process. *Korean J Intern Med* 2011; **26**: 113.

18 Zhang L, Liu T. Clinical implication of alterations in serum Klotho levels in patients with type 2 diabetes mellitus and its associated complications. *J Diabetes Complications* 2018; **32**: 922–930.

19 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.

20 Fullmer, S, Benson-Davies, S, Earthman, CP, Frankenfield, DC, Gradwell, E, Lee, P, Trabulsi J, Fullmer S, Benson-Davies S, Earthman CP, Frankenfield DC, Gradwell E *et al.* Evidence analysis library review of best practices for performing indirect calorimetry in healthy and non-critically ill individuals. *J Acad Nutr Diet* 2015; **115**: 1417–1446.e2.

21 Sundström M, Tjäder I, Rooyackers O, Wernerman J. Indirect calorimetry in mechanically ventilated patients. A systematic comparison of three instruments. *Clin Nutr* 2013; **32**: 118–21.

22 Amaro-Gahete FJ, Sanchez-Delgado G, Alcantara JMA, Martinez-Tellez B, Muñoz-Hernandez V, Merchan-Ramirez E *et al.* Congruent Validity of Resting Energy Expenditure Predictive Equations in Young Adults. *Nutrients* 2019; **11**: 1–13.

23 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.

24 Alcantara JMA, Sanchez-Delgado G, Martinez-Tellez B, Merchan-Ramirez E, Labayen I, Ruiz JR. Congruent validity and inter-day reliability of two breath by breath metabolic carts to measure resting metabolic rate in young adults. *Nutr Metab Cardiovasc Dis* 2018; **28**: 929–936.

25 Sanchez-Delgado G, Alcantara JMA, Ortiz-Alvarez L, Xu H, Martinez-Tellez B, Labayen I *et al.* Reliability of resting metabolic rate measurements in young adults: Impact of methods for data analysis. *Clin Nutr* 2017.

26 Weir J. New methods for calculating metabolic rate with special reference to protein metabolism. *J Physiol* 1949; **109**: 1–9.

27 Frayn KN. Calculation of substrate oxidation rates in vivo from gaseous exchange. *J Appl Physiol* 1983; **55**: 628–34.

28 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; : 1–20.

29 Amaro-Gahete FJ, Ruiz JR. Methodological issues related to maximal fat oxidation rate during exercise : Comment on: Change in maximal fat

oxidation in response to different regimes of periodized high-intensity interval training (HIIT). *Eur J Appl Physiol* 2018; **118**: 2029–2031.

30 Amaro-Gahete FJ, Sanchez-Delgado G, Alcantara JMA, Martinez-Tellez B, Acosta FM, Helge JW *et al.* Impact of data analysis methods for maximal fat oxidation estimation during exercise in sedentary adults. *Eur J Sport Sci* 2019; 1–10.

31 Balke B, Ware RW. An experimental study of physical fitness of Air Force personnel. *U S Armed Forces Med J* 1959; **10**: 675–88.

32 Ravussin E, Lillioja S, Anderson TE, Christin L, Bogardus C. Determinants of 24-hour energy expenditure in man. Methods and results using a respiratory chamber. *J Clin Invest* 1986; **78**: 1568–78.

33 Manini TM. Energy expenditure and aging. *Ageing Res Rev* 2010; **9**: 1–11.

34 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.

35 Rolfe DF, Brand MD. Contribution of mitochondrial proton leak to skeletal muscle respiration and to standard metabolic rate. *Am J Physiol* 1996; **271**: C1380-9.

36 Goodpaster BH, Sparks LM. Metabolic Flexibility in Health and Disease. *Cell Metab* 2017; **25**: 1027–1036.

37 Calles-Escandon J, Arciero PJ, Gardner AW, Bauman C, Poehlman ET. Basal fat oxidation decreases with aging in women. *J Appl Physiol* 1995; **78**: 266–271.

38 Solomon TPJ, Marchetti CM, Krishnan RK, Gonzalez F, Kirwan JP. Effects of aging on basal fat oxidation in obese humans. *Metabolism* 2008; **57**: 1141–1147.

39 Kim J-H, Hwang K-H, Park K-S, Kong ID, Cha S-K. Biological role of anti-aging protein Klotho. *J lifestyle Med* 2015; **5**: 1–6.

40 Kuro-o M. The Klotho proteins in health and disease. *Nat Rev Nephrol* 2019; **15**: 27–44.

41 Arking DE, Krebsova A, Macek M, Macek M, Arking A, Mian IS *et al.* Association of human aging with a functional variant of klotho. *Proc Natl Acad Sci U S A* 2002; **99**: 856–61.

42 Arking DE, Becker DM, Yanek LR, Fallin D, Judge DP, Moy TF *et al.* KLOTHO allele status and the risk of early-onset occult coronary artery disease. *Am J Hum Genet* 2003; **72**: 1154–61.

43 Ogata N, Matsumura Y, Shiraki M, Kawano K, Koshizuka Y, Hosoi T *et al.* Association of klotho gene polymorphism with bone density and spondylosis of the lumbar spine in postmenopausal women. *Bone* 2002; **31**: 37–42.

44 Arking DE, Atzmon G, Arking A, Barzilai N, Dietz HC. Association between a functional variant of the KLOTHO gene and high-density lipoprotein cholesterol, blood pressure, stroke, and longevity. *Circ Res* 2005; **96**: 412–8.

45 Hu J, Shi M, Zhang J, Quiñones H, Griffith C, Kuro-o M. Klotho deficiency causes

vascular calcification in chronic kidney disease. *J Am Soc Nephrol* 2011; **22**: 124–36.

46 Xuan NT, Hai N Van. Changes in expression of klotho affect physiological processes, diseases, and cancer. *Iran J Basic Med Sci* 2018; **21**: 3–8.

47 Zhou X, Chen K, Lei H, Sun Z. Klotho gene deficiency causes salt-sensitive hypertension via monocyte chemotactic protein-1/CC chemokine receptor 2-mediated inflammation. *J Am Soc Nephrol* 2015; **26**: 121–32.

48 Zhou L, Li Y, Zhou D, Tan RJ, Liu Y. Loss of Klotho contributes to kidney injury by depression of Wnt/catenin signaling. *J Am Soc Nephrol* 2013; **24**: 771–785.

49 Zeldich E, Chen C, Colvin TA, Bove-fenderson EA, Liang J, Zhou TBT *et al.* The Neuroprotective Effect of Klotho is Mediated via Regulation of Members of the Redox System. *J Biol Chem* 2014; **289**: 24700–24715.

50 Yamamoto M, Clark J, JV P, Gurnani P, Nandi A, Kurosu H. Regulation of oxidative stress by the anti-aging hormone klotho. *Biol Chem* 2005; **280**: 3829–3824.

Chapter 8:
Relationship of S-Klotho and cardiometabolic risk in sedentary middle-aged adults: the FIT-AGEING study
(Study 12)

ABSTRACT

This study aimed to investigate the relationship of S-Klotho with cardiometabolic risk factors in healthy sedentary middle-aged adults.

A total of 74 participants (~50% women) were included in the current cross-sectional study. A sex-specific cardiometabolic risk score was calculated based on clinical parameters selected by the International Diabetes Federation to define cardiometabolic risk including waist circumference, blood pressure, plasma glucose, HDL-C and triglycerides. S-Klotho was determined according to a solid-phase sandwich enzyme-linked immunosorbent assay kit.

There was a significant negative association between S-Klotho and cardiometabolic risk score in both men and women (β=-0.658, R2=0.433, P<0.001 and β=-0.442, R2=0.195, P=0.007) which persisted after adjusting for age, energy intake, and VO_2max.

In conclusion, the findings of the present study are that higher levels of S-Klotho were associated with lower cardiometabolic risk, and with higher insulin sensitivity in healthy sedentary middle-aged adults. Therefore, S-Klotho would be considered a biomarker of cardiometabolic health in sedentary middle-aged individuals free of diseases.

BACKGROUND

The shed form of the α-Klotho protein is thought to prevent some of the deleterious consequences of the ageing process, increasing life expectancy when is overexpressed and inducing premature ageing phenotypes when is downregulated [1-3]. Several studies have investigated the physiological mechanisms that explain the anti-ageing properties of S-Klotho discovering that it regulates the mineral homeostasis, reduces cellular oxidative stress, and attenuates chronic inflammation processes [1,4,5]. Therefore, it seems clear that the longevity effects attributed to S-Klotho could be explained for its metabolic functions. The ageing process is characterized for an increased incidence of cardiometabolic diseases, which are mainly derived from the progressive decline of several physiological functions in humans [6]. These display the major cause of morbi-mortality in developed countries [7]. Indeed, the World Health Organization reported that a total of 17.9 million of people die each year as a consequence of cardiometabolic diseases [8], and over a billion people in the world suffer from cardiometabolic diseases [9]. Evidence from clinical and experimental studies have reported that oxidative stress and chronic inflammation are closely associated with cardiometabolic disorders (i.e. obesity, type II diabetes mellitus or hypertension) [10,11]. Preserving physiological functions that control cellular oxidative stress and chronic inflammation could be determinant to reduce cardiometabolic risk during the ageing process [12].

Taking into consideration the S-Klotho physiological functions and the physio-pathological mechanisms involved in the development of cardiometabolic diseases, it biologically plausible that S-Klotho exerts a protective role against cardiometabolic risk factors. Little is known, however, about the relationship of the S-Klotho and cardiometabolic risk in humans. It has been reported that S-Klotho was inversely associated with the prevalence of cardiometabolic diseases in older adults [13]. Furthermore, low S-Klotho was related to the development of type II diabetes mellitus in human adults of both sexes [14], an unhealthy body composition status [15], poor physical fitness levels [16], and a higher risk of all-cause mortality [17].

Considering that the problem with studying the ageing process in elderly populations involves that the majority of people display ageing-related diseases [18], it is of clinical interest to investigate these physiological mechanisms in healthy and relatively young individuals aiming to reverse or delay ageing-related diseases [19]. Therefore, this study aimed to investigate the relationship of S-Klotho and cardiometabolic risk factors in healthy sedentary middle-aged adults.

MATERIAL & METHODS

Study design and participants

A total of 74 participants (~50% women) were included in the current cross-sectional study under the framework of a randomized controlled trial (FIT-AGEING project; clinicaltrial.gov: ID: NCT03334357) [20]. They were recruited via social networks, electronic media, and leaflets. Details about inclusion and exclusion criteria have been described previously [21]. Briefly, the participants were healthy individuals aged 45 to 65 years, and reported not to be physically active (<20 minutes of moderate-vigorous physical activity <3 days/week) and to have a stable weight (<3 kg changes during the last 12 weeks before the assessment). The study protocol and methodology were designed according to the last revised Declaration of Helsinki (2013). The Human Research Ethics Committee of the "Junta de Andalucía" approved the study [0838-N-2017] and all participants signed a written informed consent.

Procedures

Anthropometry

The BMI was calculated from the weight and height measurements (Seca 760, Electronic Column Scale, Hamburg, Germany) as the weight (kg) divided by the square of the height (m^2). The WC was assessed at the mid-point between the bottom of the rib cage and the iliac crest at the end of a normal expiration following standard procedures provided by the International Society for the Advancement of Kinanthropometry [22]. Three WC measurements were taken and the mean of these values was considered for statistical analysis.

Blood pressure

It was determined after resting for 30 minutes on the right arm and in a supine position. An automatic monitor (Omrom® HEM 705 CP, Health-care Co, Kyoto, Japan) was used strictly following the guidelines of the European Heart Society [23]. We measured blood pressure three times 1 minute apart, and the mean of these values was considered for the statistical analysis.

Blood samples

They were obtained from the antecubital vein after overnight fasting and resting (at least 10 minutes before) in a supine position. Blood samples were collected in prechilled EDTA-containing tubes (Vacutainer SST, Becton Dickinson, Plymouth, UK), and were immediately stored at −80°C until further use. Laboratory data included S-Klotho, glucose, insulin, total cholesterol, triglycerides, HDL-C, LDL-C, ALT, and γ-GT. S-Klotho was determined according to a solid-phase sandwich ELISA kit (Demeditec, Kiel, Germany), strictly following the manufacturer´s instruction. To determine intra- and inter-assay coefficients of variation, two different doses of purified S-Klotho were measured. We obtained that both CVs ranged from 3% to 10%. Glucose and insulin were assessed by spectrophotometrical techniques (AU5800, Beckman Coulter, Brea, California, USA) and by chemiluminescence immunoassay with paramagnetic particles (UniCel DxI 800, Beckman Coulter, Brea, California, USA), respectively. Total cholesterol, HDL-C, and triglycerides were measured by spectrophotometrical techniques automatically (AU5800, Beckman Coulter, Brea, California, USA), and LDL-C was calculated as: (Total cholesterol) – (HDL-C) – 0.45 * (Triglycerides). ALT and γ-GT were determined by absorptionspectrophotometrical techniques (Beckman Coulter, Brea, California, USA). We also calculated the insulin glucose ratio (insulin divided by glucose), the LDL-C/HDL-C ratio (LDL-C divided by HDL-C), and the triglycerides/HDL-C ratio (triglycerides divided by HDL-C).

Cardiometabolic risk indexes

A sex-specific cardiometabolic risk score was calculated based on clinical parameters selected by the International Diabetes Federation to define cardiometabolic risk including WC, blood pressure, plasma glucose, HDL-C and triglycerides [24]. These parameters were standardized as: value = (value-mean) / SD. In order to confer greater risk with increasing values, the standardized HDL-C values were multiplied by -1. Cardiometabolic risk score was calculated as the sum of these 5 standardized values divided by 5, obtaining a mean of 0 and a SD of 1 by definition, understanding lower values as a better cardiometabolic risk profile. Insulin sensitivity and resistant were calculated through the QUICKI [25] and the HOMA [26], respectively:

$$QUICKI = \frac{1}{Loge\ (Insulin) + \ Loge\ (Glucose)}$$

$$HOMA = \frac{Insulin * \ Glucose}{22.5}$$

We calculated the fatty liver index, which is a surrogate marker of fatty liver function in non-alcoholic individuals, using data of BMI, WC, triglycerides, and γ-GT, and applying a previously validated equation [27]:

$$Fatty\ liver\ index = \left(e^{\substack{0.953*loge(triglycerides) \\ +0.139* \\ body\ mass\ index+ \\ 0.718*loge(\gamma-\mathrm{GT})+0.053* \\ \text{waist circunference}-15.745)}} \right) * 100$$

Dietary intake

We assessed dietary intake through three non-consecutive 24-hours recalls determining energy, fat, carbohydrate and protein intakes [28]. The EvalFINUT® software was used to obtain these data.

Sedentary behaviour and physical activity levels

We objectively assessed sedentary behaviour and physical activity levels by triaxial accelerometry (ActiGraph GT3X+, Pensacola, FL, US) using an accelerometer in the participant´s non-dominant wrist 24-

hours/day during 7 consecutive days. Data were exported using a specific software (ActiLife v. 6.13.3, ActiGraph, Pensacola, FL, US), and processed with the GGIR package (v. 1.6-0, https://cran.r-project.org/web/packages/GGIR/index.html) in R software (v. 3.1.2, https://www.cran.r-project.org/) [29]. Sedentary time and MVPA levels were computed.

Cardiorespiratory fitness

We determined VO_2max by IC (Medgraphics Corp, Minnesota, USA) using a maximum treadmill graded exercise test extensively described elsewhere [21,30]. In brief, the participants walked at 5.3 km/h increasing the slope 1% each minute until the volitional extenuation was reached. The participants were instructed to fast for 3 hours, not to consume any drugs during the previous 48 hours, and not to perform any moderate and/or vigorous physical activity before the test (24 hours and 48 hours, respectively). The VO_2max criteria were: (i) to attain a RER ≥ 1.1, (ii) to observe a plateau in VO_2 (change of less than 100 ml/min in the last 30 s), to reach a heart rate between 10 beats/min of the age-predicted maximal heart rate. We considered the peak oxygen uptake value during the exercise test if these criteria were not met [31].

Statistical analysis

The distribution of all variables was verified using the Shapiro-Wilk test, visual check of histograms, and Q-Q plots. The descriptive parameters are reported as mean (SD). Unpaired T-Student tests were conducted to examine differences between men and women.

Simple linear regression models (Model 0) were built to study the association of S-Klotho with cardiometabolic risk score, QUICKI and HOMA. Multiple linear regression models were also performed to test these associations after adjusting by age (Model 1), by energy intake (Model 2) and by VO_2max (Model 3). Similar analyses were conducted to study the association between S-Klotho with cardiometabolic risk factors. The Statistical Package for Social Sciences (SPSS, v. 22.0, IBM Corporation, Chicago, IL, USA) and the GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) were used to perform the analyses and to build graphical plots, respectively. We fixed the level of significance at <0.05.

RESULTS

Table 1 shows the descriptive characteristics of our study participants.

There was a significant negative association between S-Klotho and cardiometabolic risk score in both men and women (β=-0.658, R^2=0.433, $P<0.001$ and β=-0.442, R^2=0.195, P=0.007; Figure 1B and Figure 1C, respectively) which persisted after adjusting for age, energy intake, and VO_2max (all $P<0.05$; Figure 1B and Figure 1C).

S-Klotho was positively related to QUICKI in men (β=0.376, R^2=0.141, P=0.029; Figure 1E) as well as in women (β=0.382, R^2=0.146, P=0.016 Figure 1F), which remained significant when energy intake was included in the model as a covariate (all P<0.05; Figure 1E and Figure 1F). These associations persisted in women after adjusting for age and VO_2max (all P<0.05; Figure 1F), whereas disappeared in men (all P>0.09; Figure 1F).

We observed a significant negative association between S-Klotho and HOMA in both men and women (β=-0.377, R^2=0.142, P=0.031 and β=-0.465, R^2=0.216, P=0.003; Figure 1H and Figure 1I, respectively), which remained after adjusting for energy intake (all P<0.04; Figure 1H and Figure 1I). These results persisted in women after controlling for age and VO_2max (all P<0.03; Figure 1I), while became non-significant in men (all P>0.08; Figure 1H).

There was a significant negative association of S-Klotho with systolic and diastolic blood pressure, insulin, insulin-glucose ratio, total cholesterol, triglycerides, LDL-C, LDL-C, LDL-C/HDL-C ratio, and Triglycerides/HDL-C ratio (all P<0.05; Table 2) which remained significant after adjusting for age, energy intake, and VO_2max in both men and women (Table 2). We also observed that S-Klotho was positively related to HDL-C in both sexes (all P<0.02; Table 2), which persisted when VO_2max was included as a covariate (all P≥0.001; Table 2). These results remained significant in men after controlling for age and energy intake (all P<0.001; Table 2), whereas were attenuated in women (all P<0.1; Table 2). There was no association between S-Klotho with ALT, γ-GT and fatty liver index neither in men (all P>0.2; Table 2) nor in women (all P>0.1; Table 2).

These findings did not change after controlling for macronutrient intake (fat, carbohydrate, and protein intake), sedentary time and/or moderate-vigorous physical activity levels (data not shown).

Table 1. Descriptive characteristic of participants.

	N	All		N	Men		N	Women	
Age (years)	74	53.7	(5.1)	35	54.4	(5.3)	39	53.0	(5.0)
S-Klotho (pg/ml)	73	775.3	(363.7)	34	814.1	(452.2)	39	741.4	(265.6)
Anthropometry									
Weight (kg)	74	75.7	(15.0)	35	87.4	(11.0)	39	65.3	(9.3)*
Height (cm)	74	167.8	(9.8)	35	175.8	(6.5)	39	160.7	(6.1)*
BMI (kg/m^2)	74	26.7	(3.8)	35	28.3	(3.6)	39	25.3	(3.3)*
WC (cm)	74	95.1	(11.7)	35	102.7	(8.8)	39	88.2	(9.7)*
Blood pressure									
Systolic blood pressure (mm Hg)	70	127.1	(15.8)	32	134.3	(13.8)	38	120.9	(14.8)*
Diastolic blood pressure (mm Hg)	70	81.1	(11.7)	32	85.2	(10.9)	38	77.6	(11.4)*
Mean blood pressure (mm Hg)	70	104.1	(13.1)	32	109.7	(11.7)	38	99.3	(12.5)*
Glycaemic metabolism									
Glucose (mg/dL)	73	93.5	(11.2)	34	94.8	(13.4)	38	92.4	(8.8)
Insulin (UI/mL)	73	8.2	(5.6)	34	8.8	(6.7)	38	7.6	(4.6)
Insulin glucose ratio	73	12.7	(7.5)	34	13.2	(8.1)	38	12.3	(7.1)
QUICKI	73	0.362	(0.036)	34	0.357	(0.039)	38	0.365	(0.033)
HOMA	73	1.95	(1.65)	34	2.17	(2.09)	38	1.76	(1.14)
Lipid metabolism									
Total cholesterol (mg/dL)	73	207.5	(33.5)	34	203.8	(36.7)	39	210.6	(30.5)
Triglycerides (mg/dL)	73	136	(67.8)	34	147.4	(83.8)	39	126.1	(49.1)
HDL-C (mg/dL)	73	59.1	(12.8)	34	55.5	(12.7)	39	62.3	(12.2)*
LDL-C (mg/dL)	73	126.6	(29.3)	34	127.8	(31.9)	39	125.5	(27.2)
LDL-C/HDL-C	73	2.30	(0.91)	34	2.48	(0.96)	39	2.10	(0.80)
Triglycerides/HDL-C	73	2.58	(1.89)	34	3.05	(2.36)	39	2.17	(1.25)*
Cardiometabolic risk score	70	0.002	(0.34)	32	0.026	(0.384)	38	-0.017	(0.302)*
Liver function									
ALT (IU/L)	73	23.3	(12.5)	34	29.0	(13.6)	39	18.3	(9.0)*
γ-GT (IU/L)	73	34.4	(23.2)	34	41.0	(23.3)	39	28.7	(21.9)*
Fatty liver index	73	50.0	(26.0)	34	66.7	(20.2)	39	35.5	(21.6)
Dietary intake									
Energy (kcal/day)	73	2134	(688)	34	2374	(838)	39	1913	(414)*
Fat (g/day)	73	38.8	(10.1)	34	38.0	(5.9)	39	39.5	(12.8)
Carbohydrate (g/day)	73	52.4	(29.3)	34	49.0	(15.6)	39	55.6	(37.8)
Protein (g/day)	73	22.5	(21.0)	34	19.1	(8.8)	39	25.7	(27.7)
Ethanol (g/day)	73	11.2	(13.1)	34	16.2	(16.0)	39	6.6	(7.2)*
Sedentary behaviour and physical activity levels									
Sedentary time (min/day)	71	745.9	(84.2)	34	770.0	(80.3)	37	723.7	(82.6)*
MVPA (min/day)	71	96.1	(35.4)	34	96.6	(35.5)	37	95.5	(35.8)
Cardiorespiratory fitness									
VO_2max (ml/min)	71	2339	(657.2)	34	2915	(373.2)	37	1809	(332.5)*
VO_2max (ml/kg/min)	71	30.5	(5.6)	34	33.3	(4.5)	37	27.9	(5.3)*

Data are shown as means (SD). *Significant differences between sexes obtained from a T-Student unpaired-samples test (P<0.05). Abbreviations: S-Klotho; shed form of the Klotho protein, QUICKI; Quantitative insulin sensitivity check index, HOMA; Homeostasis model assessment index, HDL-C; High-density lipoprotein cholesterol, LDL-C; Low-density lipoprotein cholesterol, ALT; Alanine transaminase, γ-GT; γ-glutamyl transferase, MVPA; Moderate-vigorous intensity physical activity levels, VO2max; Maximal oxygen uptake.

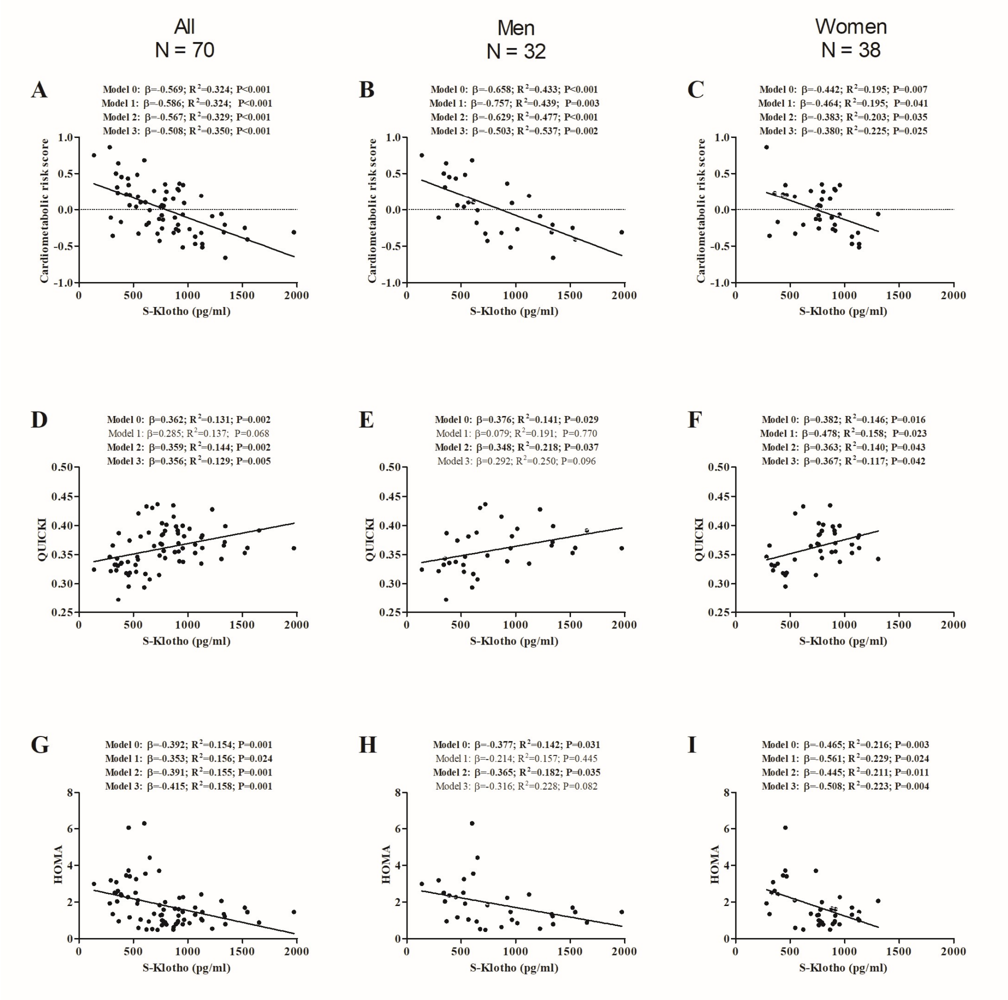

Figure 1. Association between S-Klotho with cardiometabolic risk index, fatty liver index, quantitative insulin sensitivity check index (QUICKI), and homeostatic model assessment of insulin resistance index (HOMA) in middle-age sedentary adults. β (standardized regression coefficient), R^2, and P from simple and multiple linear regression analyses. Model 0; unadjusted, Model 1; adjusted by age, Model 2; adjusted by energy intake, Model 3; adjusted by cardiorespiratory fitness

Table 2. Association between S-Klotho with cardiometabolic risk parameters (Model 0, unadjusted), adjusted by age (Model 1), adjusted by energy intake (Model 2) and adjusted by cardiorespiratory fitness (Model 3).

	All			Men			Women		
	β	R^2	P	β	R^2	P	β	R^2	P
Weight (kg)									
Model 0	**0.249**	**0.062**	**0.034**	0.294	0.087	0.091	0.210	0.044	0.200
Model 1	**0.363**	**0.076**	**0.026**	0.116	0.105	0.684	0.043	0.081	0.838
Model 2	**0.250**	**0.114**	**0.031**	0.300	0.090	0.091	0.208	0.044	0.262
Model 3	0.197	0.049	0.125	**0.491**	**0.439**	**0.002**	**0.402**	**0.273**	**0.015**
WC (cm)									
Model 0	**0.230**	**0.053**	**0.050**	0.334	0.112	0.054	0.079	0.006	0.634
Model 1	**0.325**	**0.063**	**0.046**	0.197	0.112	0.487	-0.047	0.028	0.828
Model 2	**0.229**	**0.115**	**0.047**	0.344	0.122	0.051	0.218	0.102	0.225
Model 3	0.238	0.054	0.055	**0.558**	**0.512**	**<0.001**	0.306	0.257	0.063
Systolic blood pressure (mm Hg)									
Model 0	**-0.536**	**0.287**	**<0.001**	**-0.767**	**0.588**	**<0.001**	**-0.487**	**0.237**	**0.003**
Model 1	-0.222	0.388	0.116	**-0.868**	**0.594**	**<0.001**	-0.108	0.388	0.541
Model 2	**-0.539**	**0.303**	**<0.001**	**-0.775**	**0.591**	**<0.001**	**-0.634**	**0.336**	**<0.001**
Model 3	**-0.609**	**0.325**	**<0.001**	**-0.708**	**0.603**	**<0.001**	**-0.505**	**0.240**	**0.004**
Diastolic blood pressure (mm Hg)									
Model 0	**-0.443**	**0.196**	**<0.001**	**-0.618**	**0.381**	**<0.001**	**-0.359**	**0.129**	**0.031**
Model 1	**-0.126**	**0.298**	**0.003**	**-0.565**	**0.383**	**<0.001**	-0.005	0.268	0.982
Model 2	**-0.449**	**0.202**	**<0.001**	**-0.613**	**0.382**	**<0.001**	**-0.529**	**0.247**	**0.004**
Model 3	**-0.492**	**0.213**	**<0.001**	**-0.511**	**0.431**	**0.004**	**-0.390**	**0.137**	**0.030**
Mean blood pressure (mm Hg)									
Model 0	**-0.519**	**0.269**	**<0.001**	**-0.740**	**0.547**	**<0.001**	**-0.453**	**0.205**	**0.006**
Model 1	-0.189	0.380	0.180	**-0.775**	**0.548**	**0.001**	-0.062	0.365	0.752
Model 2	**-0.523**	**0.280**	**<0.001**	**-0.742**	**0.547**	**<0.001**	**-0.616**	**0.232**	**0.001**
Model 3	**-0.585**	**0.299**	**<0.001**	**-0.655**	**0.578**	**<0.001**	**-0.478**	**0.210**	**0.006**
Glucose (mg/dL)									
Model 0	-0.024	0.001	0.839	-0.056	0.003	0.755	0.011	0.000	0.948
Model 1	-0.039	0.001	0.826	-0.043	0.003	0.886	-0.084	0.012	0.701
Model 2	-0.017	0.038	0.886	-0.025	0.094	0.883	0.007	0.004	0.971
Model 3	-0.030	0.001	0.819	-0.024	0.020	0.902	0.042	0.002	0.822
Insulin (UI/mL)									
Model 0	**-0.432**	**0.187**	**0.001**	**-0.423**	**0.179**	**0.013**	**-0.504**	**0.254**	**0.001**
Model 1	**-0.378**	**0.189**	**0.012**	-0.330	0.184	0.229	**-0.585**	**0.263**	**0.004**
Model 2	**-0.430**	**0.197**	**<0.001**	**-0.396**	**0.251**	**0.016**	**-0.480**	**0.250**	**0.005**
Model 3	**-0.470**	**0.199**	**<0.001**	**-0.391**	**0.231**	**0.031**	**-0.546**	**0.257**	**0.002**
Insulin glucose ratio									
Model 0	**-0.488**	**0.238**	**<0.001**	**-0.492**	**0.242**	**0.003**	**-0.534**	**0.285**	**<0.001**
Model 1	**-0.437**	**0.241**	**0.003**	-0.374	0.250	0.157	**-0.593**	**0.290**	**0.003**
Model 2	**-0.487**	**0.240**	**<0.001**	**-0.472**	**0.236**	**0.004**	**-0.508**	**0.242**	**0.003**
Model 3	**-0.512**	**0.244**	**<0.001**	**-0.444**	**0.319**	**0.010**	**-0.565**	**0.276**	**0.001**
Total colesterol (mg/dL)									
Model 0	**-0.631**	**0.339**	**<0.001**	**-0.704**	**0.496**	**<0.001**	**-0.516**	**0.266**	**0.001**
Model 1	**-0.647**	**0.399**	**<0.001**	**-0.497**	**0.520**	**0.022**	**-0.641**	**0.287**	**0.001**
Model 2	**-0.637**	**0.406**	**<0.001**	**-0.701**	**0.496**	**<0.001**	**-0.515**	**0.282**	**0.002**
Model 3	**-0.614**	**0.440**	**<0.001**	**-0.726**	**0.523**	**<0.001**	**-0.550**	**0.355**	**0.001**
Triglycerides (mg/dL)									
Model 0	**-0.549**	**0.301**	**<0.001**	**-0.634**	**0.401**	**<0.001**	**-0.428**	**0.183**	**0.007**
Model 1	**-0.505**	**0.304**	**<0.001**	**-0.748**	**0.409**	**<0.001**	-0.355	0.190	0.081
Model 2	**-0.548**	**0.302**	**<0.001**	**-0.613**	**0.445**	**<0.001**	-0.302	0.244	0.071
Model 3	**-0.556**	**0.297**	**<0.001**	**-0.598**	**0.390**	**0.001**	**-0.374**	**0.194**	**0.031**
HDL-C (mg/dL)									
Model 0	**0.592**	**0.350**	**<0.001**	**0.852**	**0.726**	**<0.001**	**0.378**	**0.372**	**0.020**
Model 1	**0.486**	**0.362**	**<0.001**	**0.997**	**0.738**	**<0.001**	0.266	0.153	0.196
Model 2	**0.595**	**0.390**	**<0.001**	**0.862**	**0.737**	**<0.001**	0.285	0.160	0.104
Model 3	**0.784**	**0.540**	**<0.001**	**0.917**	**0.790**	**<0.001**	**0.589**	**0.307**	**0.001**

Table 2. Continued

	All			Men			Women		
	β	R^2	P	β	R^2	P	β	R^2	P
LDL-C (mg/dL)									
Model 0	**-0.559**	**0.312**	**<0.001**	**-0.718**	**0.516**	**<0.001**	**-0.329**	**0.108**	**0.041**
Model 1	**-0.380**	**0.347**	**0.006**	**-0.396**	**0.575**	**0.050**	-0.215	0.125	0.302
Model 2	**-0.562**	**0.315**	**<0.001**	**-0.713**	**0.519**	**<0.001**	-0.267	0.122	0.098
Model 3	**-0.603**	**0.386**	**<0.001**	**-0.757**	**0.551**	**<0.001**	**-0.415**	**0.239**	**0.015**
LDL-C/HDL-C									
Model 0	**-0.657**	**0.432**	**<0.001**	**-0.858**	**0.736**	**<0.001**	**-0.444**	**0.197**	**0.005**
Model 1	**-0.512**	**0.455**	**<0.001**	**-0.736**	**0.744**	**<0.001**	-0.366	0.205	0.070
Model 2	**-0.657**	**0.439**	**<0.001**	**-0.854**	**0.737**	**<0.001**	**-0.379**	**0.212**	**0.028**
Model 3	**-0.743**	**0.508**	**<0.001**	**-0.814**	**0.730**	**<0.001**	**-0.536**	**0.131**	**0.001**
Triglycerides/HDL-C									
Model 0	**-0.564**	**0.318**	**<0.001**	**-0.654**	**0.428**	**<0.001**	**-0.480**	**0.230**	**0.002**
Model 1	**-0.485**	**0.325**	**0.001**	**-0.760**	**0.434**	**0.002**	-0.374	0.245	0.058
Model 2	**-0.564**	**0.318**	**<0.001**	**-0.637**	**0.456**	**<0.001**	**-0.357**	**0.287**	**0.029**
Model 3	**-0.620**	**0.347**	**<0.001**	**-0.650**	**0.430**	**<0.001**	**-0.481**	**0.268**	**0.005**
ALT (IU/L)									
Model 0	0.100	0.010	0.399	0.186	0.035	0.292	-0.209	0.044	0.201
Model 1	0.148	0.013	0.371	0.237	0.036	0.424	-0.473	0.138	0.226
Model 2	0.102	0.059	0.385	0.182	0.036	0.313	-0.161	0.049	0.383
Model 3	0.035	0.065	0.781	0.137	0.027	0.485	-0.094	0.009	0.614
γ-GT (IU/L)									
Model 0	0.110	0.012	0.354	0.194	0.038	0.272	-0.075	0.006	0.648
Model 1	0.059	0.015	0.720	0.238	0.039	0.422	-0.347	0.106	0.103
Model 2	0.112	0.023	0.349	0.199	0.041	0.268	-0.030	0.012	0.871
Model 3	0.074	0.054	0.562	0.225	0.044	0.250	-0.082	0.039	0.653
Fatty liver index									
Model 0	0.106	0.011	0.373	0.138	0.019	0.437	-0.048	0.002	0.771
Model 1	0.157	0.014	0.342	-0.076	0.045	0.797	-0.223	0.044	0.305
Model 2	0.108	0.045	0.364	0.159	0.063	0.371	0.063	0.057	0.732
Model 3	0.133	0.016	0.307	-0.358	0.442	0.148	0.180	0.195	0.285

P value of multiple-regression analysis. β (standardized regression coefficient). Abbreviations: S-Klotho; shed form of the Klotho protein, HDL-C; High-density lipoprotein cholesterol, LDL-C; Low-density lipoprotein cholesterol, ALT; Alanine transaminase, γ-GT; γ-glutamyl transferase

DISCUSSION

The main findings of the present study are that S-Klotho was inversely associated with cardiometabolic risk and insulin resistance in both sedentary men and women independently of age, cardiorespiratory fitness, physical activity levels, and dietary intake. These findings support the idea that S-Klotho could be a good indicator of cardiometabolic status in healthy sedentary middle-aged individuals.

The discovery of the α-Klotho gene is relatively new [2] and numerous studies have investigated its genetic regulation and physiological functions. However, whether S-Klotho is related to cardiometabolic risk has not extensively studied. Semba et al. [13,17] reported a strong association between higher S-Klotho with lower likelihood of cardiovascular disease and mortality risk in a large cohort of elderly adults greater than 65 years. Similarly, the results of a recent study showed that patients with coronary artery disease present lower S-Klotho, as well as a reduced α-Klotho gene expression in the vascular wall [32]. Kitagawa et al. [33] observed that decreases in S-Klotho were independently associated with signs of vascular dysfunction such as arterial stiffness in patients with chronic kidney disease. Indeed, it has been suggested S-Klotho as a promising diagnostic biomarker or as a therapeutic factor for the treatment of cardiovascular diseases [34]. In contrast, Branderburg et al. [35] suggested that S-Klotho was not related to cardiovascular and all-cause mortality in a cohort with normal and mildly impaired renal function but at high risk for future cardiovascular events [35]. These discrepancies could be explained by the different health status and age of the participants involved in each study, which could involve a wide range of physiological impairments. However, to the best of our knowledge, there are no studies investigating the association of S-Klotho with cardiometabolic risk in healthy sedentary middle-aged adults of both sexes. Our results suggest a positive association of S-Klotho and decreased cardiometabolic risk in this population cohort, supporting the idea that S-Klotho could be a good indicator of cardiometabolic health in individuals free of disease.

The cardiometabolic protective role of S-Klotho could be explained for its metabolic functions. S-Klotho attenuates vascular calcification and exerts vasoprotective effects increasing the nitric oxide production via upregulating the endothelial nitric oxide synthase activity [1], maintaining endothelial homeostasis [36]. S-Klotho is also a phosphaturic hormone, which functions as a β-glucuronidase able to modify the NaPi-2A gene expression and inducing phosphaturia [37]. In this way, a reduction of phosphate levels prevents vascular calcification and cardiovascular impairments [1]. Furthermore, S-Klotho downregulates the production of pro-inflammatory cytokines [36]. Given that it has previously shown that a chronic inflammatory status is closely linked with the development of cardiometabolic diseases (i.e. metabolic syndrome and/or type II diabetes mellitus among others) [38], the reduction of pro-inflammatory cytokines induced by S-Klotho may decrease cardiometabolic risk.

There is considerable evidence that low S-Klotho is strongly associated with the development of type 2 diabetes mellitus in prediabetic patients [14], greater insulin resistance in type 2 diabetes mellitus patients [39], and increased type 2 diabetes mellitus complications (i.e. diabetic nephropathy or diabetic coronary heart disease among others) [40,41]. However, there is a lack of evidence investigating whether S-Klotho is linked with insulin sensitivity/resistance in healthy populations with the absence of chronic cardiometabolic diseases. Our results showed that S-Klotho was associated with insulin sensitivity in both healthy men and women independently of several confounder factors, thus it seems plausible to think that S-Klotho plays an important role in glycaemic and lipid metabolism. Previous studies have reported that the S-Klotho protein (i) inhibits the PI3K activation, and the insulin/IGF-I receptors [1,36], (ii) downregulates the production of pro-inflammatory cytokines [36], and (iii) attenuates oxidative stress via increasing the FOXO transcription factors that elevates catalase and mitochondrial manganese-superoxide dismutase activity [42]. Considering that these physiological mechanisms contribute with the improvement of insulin sensitivity [43,44], S-Klotho would be considered as a protective hormone against the development of insulin resistance in healthy individuals.

Limitations

Some potentials limitations of our study need to be acknowledged. Given that our study presents a cross-sectional design, no causal interpretation can be established. S-Klotho might not sufficiently reflect tissue levels of Klotho protein which cannot be obtained and analyzed in the absence of a clinical indication for biopsy. Our participants were sedentary middle-aged adults (45-65 years old), and we do not know whether these results can be extended to younger, older or physically active individuals. Additionally, based on the inclusion criteria of the present study, we cannot assess the impact of S-Klotho upon mortality in a diseased population, since our data only allow conclusions about their cardioprotective effects on healthy people.

CONCLUSIONS

In conclusion, the findings of the present study are that S-Klotho was negatively associated with cardiometabolic risk, and positively related to insulin sensitivity/resistance in healthy sedentary middle-aged adults independently of potential confounders such us age, cardiorespiratory fitness, physical activity levels and dietary intake. Therefore, S-Klotho would be considered a biomarker of cardiometabolic health in sedentary middle-aged individuals free of diseases. Further studies are needed to examine whether changes in cardiovascular risk in response to different public health interventions (i.e. physical exercise or nutritional strategies) are mediated by changes in S-Klotho.

Clinical implications

We show, for the first time, that high S-Klotho is associated with better cardiometabolic status in healthy sedentary middle-aged adults. These results have important clinical implications and support the idea that S-Klotho would play an important role in cardiometabolic processes exerting a protective role. S-Klotho has been previously postulated as a longevity biomarker [3], a cancer biomarker [45], a chronic kidney disease biomarker [46], and an acute kidney injury biomarker [47,48]. Our findings suggest that S-Klotho could also be a good biomarker of the cardiometabolic status in healthy population. Therefore, it would be of clinical interest to establish references values of S-Klotho in both healthy individuals and patients of different ages as an indicator of cardiometabolic health.

REFERENCES

1 Kuro-o M. The Klotho proteins in health and disease. *Nat Rev Nephrol* 2019; **15**: 27–44.

2 Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T *et al.* Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* 1997; **390**: 45–51.

3 Koyama D, Sato Y, Aizawa M, Maki T, Kurosawa M, Kuro-o M *et al.* Soluble αKlotho as a candidate for the biomarker of aging. *Biochem Biophys Res Commun* 2015; **467**: 1019–1025.

4 Xu Y, Sun Z. Molecular basis of klotho: From gene to function in aging. *Endocr Rev* 2015; **36**: 174–193.

5 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.

6 North BJ, Sinclair DA. The intersection between aging and cardiovascular disease. *Circ Res* 2012; **110**: 1097–1108.

7 Ho JY, Hendi AS. Recent trends in life expectancy across high income countries: Retrospective observational study. *BMJ* 2018; **362**.

8 WHO. Cardiovascular diseases (CVDs). 2018.

9 Saklayen MG. The global epidemic of the metabolic syndrome. *Curr Hypertens Rep* 2018; **20**: 12.

10 Lopez-Candales A, Hernández Burgos PM, Hernandez-Suarez DF, Harris D. Linking Chronic Inflammation with Cardiovascular Disease: From Normal Aging to the Metabolic Syndrome. *J Nat Sci* 2017; **3**.

11 Sverdlov AL, Figtree GA, Horowitz JD, Ngo DTM. Interplay between Oxidative Stress and Inflammation in Cardiometabolic Syndrome. *Mediators Inflamm* 2016; **2016**: 8254590.

12 Guarner V, Rubio-ruiz ME. Aging , Metabolic Syndrome and the Heart. *Aging Dis* 2012; **3**: 269–279.

13 Semba RD, Cappola AR, Sun K, Bandinelli S, Dalal M, Crasto C *et al.* Plasma klotho and cardiovascular disease in adults. *J Am Geriatr Soc* 2011; **59**: 1596–601.

14 Zhang L, Liu T. Clinical implication of alterations in serum Klotho levels in patients with type 2 diabetes mellitus and its associated complications. *J Diabetes Complications* 2018; **32**: 922–930.

15 Amaro-Gahete FJ, De-la-O A, Jurado-Fasoli L, Espuch-Oliver A, de Haro T, Gutierrez A *et al.* Body composition and S-Klotho plasma levels in middle-aged adults: A cross-sectional study. *Rejuvenation Res* 2019; 1–16.

16 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 sedentary middle-aged adults: the FIT-AGEING study. *Maturitas* 2019.

17 Semba RD, Cappola AR, Sun K, Bandinelli S, Dalal M, Crasto C *et al.* Plasma klotho

and mortality risk in older community-dwelling adults. *Journals Gerontol - Ser A Biol Sci Med Sci* 2011; **66 A**: 794–800.

18 Fulop T, Larbi A, Witkowski JM, McElhaney J, Loeb M, Mitnitski A *et al.* Aging, frailty and age-related diseases. *Biogerontology* 2010; **11**: 547–563.

19 Fontana L, Kennedy BK, Longo VD. Medical research: Treat ageing. *Nature* 2014; **511**: 405–406.

20 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.

21 Amaro-Gahete F, De-la-O A, Jurado-Fasoli L, Espuch-Oliver A, de Haro T, Gutiérrez A *et al.* Exercise training increases the S-Klotho plasma levels in sedentary middle-aged adults: a randomised controlled trial. *J Sport Sci* 2019.

22 Norton K, Whittingham N, Carter L, Kerr D, Gore C, Marfell-Jones M. *Measurement techniques in anthropometry*. Anthropome. 1996.

23 Whelton PK, Williams B. The 2018 European Society of Cardiology/European Society of Hypertension and 2017 American College of Cardiology/American Heart Association Blood Pressure Guidelines. *JAMA* 2018; **320**: 1749.

24 Carracher AM, Marathe PH, Close KL. International Diabetes Federation 2017. *J Diabetes* 2018; **10**: 353–356.

25 Katz A, Nambi SS, Mather K, Baron AD, Follmann DA, Sullivan G *et al.* Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. *J Clin Endocrinol Metab* 2000; **85**: 2402–10.

26 Ascaso JF, Romero P, Real JT, Priego A, Valdecabres C, Carmena R. [Insulin resistance quantification by fasting insulin plasma values and HOMA index in a non-diabetic population]. *Med Clin (Barc)* 2001; **117**: 530–3.

27 Bedogni G, Bellentani S, Miglioli L, Masutti F, Passalacqua M, Castiglione A *et al.* The Fatty Liver Index: a simple and accurate predictor of hepatic steatosis in the general population. *BMC Gastroenterol* 2006; **6**: 33.

28 Baugh E, Hedrick VE, Marinik EL, Kyle D. Dietary Intake Modification in Response to Participation in a Resistance Training Program for Sedentary Older Adults with Prediabetes: Findings from the Resist Diabetes Study. *Eat Behav* 2015; **15**: 379–382.

29 Acosta FM, Martinez-Tellez B, Sanchez-Delgado G, Migueles JH, Contreras-Gomez MA, Martinez-Avila WD *et al.* Association of Objectively Measured Physical Activity With Brown Adipose Tissue Volume and Activity in Young Adults. *J Clin Endocrinol Metab* 2019; **104**: 223–233.

30 Amaro-Gahete F, De-la-O A, Jurado-Fasoli L, Dote-Montero M, Gutiérrez A, Ruiz J *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 randomised controlled trial. *Front Physiol* 2019.

31 Midgley AW, McNaughton LR, Polman R, Marchant D. Criteria for determination of maximal oxygen uptake: A brief critique and recommendations for future research. *Sport Med* 2007; **37**: 1019–1028.

32 Navarro-González JF, Donate-Correa J, De Fuentes MM, Pérez-Hernández H, Martínez-Sanz R, Mora-FernáNdez C. Reduced Klotho is associated with the presence and severity of coronary artery disease. *Heart* 2014; **100**: 34–40.

33 Kitagawa M, Sugiyama H, Morinaga H, Inoue T, Takiue K, Ogawa A *et al.* A Decreased Level of Serum Soluble Klotho Is an Independent Biomarker Associated with Arterial Stiffness in Patients with Chronic Kidney Disease. *PLoS One* 2013; **8**.

34 Martín-Núñez E. Implications of Klotho in vascular health and disease. *World J Cardiol* 2014; **6**: 1262.

35 Brandenburg VM, Kleber ME, Vervloet MG, Larsson TE, Tomaschitz A, Pilz S *et al.* Soluble klotho and mortality: The Ludwigshafen Risk and Cardiovascular Health Study. *Atherosclerosis* 2015; **242**: 483–489.

36 Dalton GD, Xie J, An SW, Huang CL. New insights into the mechanism of action of soluble klotho. *Front Endocrinol (Lausanne)* 2017; **8**: 1–10.

37 Hu M, Shi M, Zhang J, Pastor J, Nakatani T, Lanske B *et al.* Klotho: a novel phosphaturic substance acting as an autocrine enzyme in the renal proximal tubule. *FASEB J* 2010; **24**: 3438–3450.

38 Park HK, Kwak MK, Kim HJ, Ahima RS. Linking resistin, inflammation, and cardiometabolic diseases. *Korean J Intern Med* 2017; **32**: 239–247.

39 Silva AP, Mendes F, Pereira L, Fragoso A, Gonçalves RB, Santos N *et al.* Klotho levels: association with insulin resistance and albumin-to-creatinine ratio in type 2 diabetic patients. *Int Urol Nephrol* 2017; **49**: 1809–1814.

40 Asai O, Nakatani K, Tanaka T, Sakan H, Imura A, Yoshimoto S *et al.* Decreased renal α-Klotho expression in early diabetic nephropathy in humans and mice and its possible role in urinary calcium excretion. *Kidney Int* 2012; **81**: 539–547.

41 Nie F, Wu D, Du H, Yang X, Yang M, Pang X *et al.* Serum klotho protein levels and their correlations with the progression of type 2 diabetes mellitus. *J Diabetes Complications* 2017; **31**: 594–598.

42 Yamamoto M, Clark JJD, Pastor J V., Gurnani P, Nandi A, Kurosu H *et al.* Regulation of oxidative stress by the anti-aging hormone klotho. *Biol Chem* 2005; **280**: 3829–3824.

43 Yaribeygi H, Farrokhi FR, Butler AE,

Sahebkar A. Insulin resistance: Review of the underlying molecular mechanisms. *J Cell Physiol* 2019; **234**: 8152–8161.

44 Petersen MC, Shulman GI. Mechanisms of Insulin Action and Insulin Resistance. *Physiol Rev* 2018; **98**: 2133–2223.

45 Zhou X, Wang X. Klotho: a novel biomarker for cancer. *J Cancer Res Clin Oncol* 2015; **141**: 961–969.

46 Castaño A, Maurer MS. Potential Application of Klotho in Human Chronic Kidney Disease. *Bone* 2017; **100**: 41–49.

47 Hu M, Moe OW. Klotho as a potential biomarker and therapy for acute kidney injury. *Nat Rev Nephrol* 2012; **8**: 423–429.

48 Liu YJ, Sun HD, Chen J, Chen MY, Ouyang B, Guan XD. Klotho: A novel and early biomarker of acute kidney injury after cardiac valve replacement surgery in adults. *Int J Clin Exp Med* 2015; **8**: 7351–7358.

SECTION 3: Role of exercise on S-Klotho protein, physical fitness, energy metabolism and cardiometabolic health

This section includes a total of five chapters aiming to study the effects of different exercise training modalities on S-Klotho, physical fitness, energy metabolism, and cardiometabolic health in sedentary middle-aged adults. Concretely, it has been study the effects of PAR, HIIT and HIIT+EMS interventions on: [i] S-Klotho (**Study 13**), [ii] on body composition **(Study 14)**, [iii] on physical fitness **(Study 15), [iv]** on BMR and fat oxidation, in basal conditions and during exercise **(Study 16)**, and [v] on cardiometabolic risk **(Study 17)** in sedentary middle-aged adults.

Chapter 9:
Exercise training increases the S-Klotho in sedentary middle-aged adults: a randomised controlled trial
(Study 13)

ABSTRACT

This study aimed to investigate the effects of different training modalities on the soluble Klotho (S-Klotho) plasma levels in sedentary middle-aged adults.

A total of 74 middle-aged adults (53.4±5.0 years old; 52.7% women) were enrolled in the FIT-AGEING study. We conducted a 12-week randomised controlled trial. The participants were randomly assigned to 4 different groups: (i) a control group (no exercise), (ii) a PAR group, (iii) a HIIT group, and (iv) a HIIT-EMS group. S-Klotho, anthropometric measurements, and body composition variables were measured before and after the intervention programme. All exercise training modalities induced an increase in S-Klotho (all P≤0.019) without statistical differences between them (all P≥0.696). We found a positive association between changes in LMI and changes in the S-Klotho, whereas a negative association was reported between changes in FM outcomes and changes in the S-Klotho after our intervention study.

In conclusion, our results suggest that the link between exercise training and the increase in S-Klotho could be mediated by a decrease of FM and an increase of LM.

BACKGROUND

Increasing the amount of exercise improves physical and mental health of healthy people effectively, and prevents the development of many chronic diseases [1]. Exercise is an excellent therapeutic intervention for obesity, cardiovascular disease, type 2 diabetes, certain types of cancer, and many other chronic diseases [2]. It also has an impact on life expectancy [3], yet the physiological mechanisms that may mediate these effects are not fully understood.

The Klotho gene was identified 20 years ago as a gene mutated in a mouse strain. It displayed an extremely shortened life span with multiple disorders resembling human premature-ageing syndromes that included infertility, atherosclerosis, skin atrophy, osteoporosis, emphysema, muscle atrophy, sarcopenia, and cardiovascular disease [4,5]. Other studies observed that Klotho overexpression was associated with a significantly longer lifespan [6], an increase of stem cell numbers concerned in the regenerative response [7], and an inhibition of cancer development and progression [8].

Three Klotho gene products have been identified: α-Klotho (which is expressed in distal convoluted tubules in the kidney, parathyroid and choroid plexus in the brain), β-Klotho (which control the bile acids, lipid and energy metabolism together with FGF15/19, and FGF21) and γ-Klotho (which is involved in brown adipose tissue metabolism) [9,10]. They share a substantial degree of homology, but they also seem to have different physiological actions [9,10]. The α-Klotho gene encodes a type 1 single-pass transmembrane glycoprotein [4]. The intracellular domain is short and non-functional [10]. The extracellular domain, however, forms a complex with FGF23 and FGFR1 [10] and has a potential site for proteolytic cleavage [11,12]. The cleaved Klotho is commonly known as S-Klotho, and it is detected in blood, urine, and cerebrospinal fluid [6,13]. S-Klotho can act as a soluble paracrine or endocrine mediator through the modulation of the action of growth factors and cytokines such as insulin, IGF-I (acting as a suppressor of tyrosine phosphorylation of insulin and IGF-I receptors, which results in reduced activity of insulin receptor substrate proteins and their association with PI3K, thereby inhibiting insulin and IGF-1), TGF-β, Wnt signalling, and IFNγ, which are associated with cell senescence and the ageing process in mice [10,13,14]. Indeed, higher S-Klotho has been associated with improved survival in chronic kidney disease patients [15], and lower levels have been related to increased cardiovascular disease incidence in adults [16] and all-cause mortality in chronic haemodialysis patients [17].

Although the role of exercise in the ageing process is well established [2,3], few studies have analysed the acute and chronic effects of exercise on S-Klotho [18]. Some studies reported significant increases in S-Klotho after a single bout of exercise, concluding that healthy young well-trained individuals registered a greater improvement than the elderly untrained counterparts [19–22]. We only found one manuscript that described the chronic effect on S-Klotho after 12 weeks of low-to-moderate intensity aerobic training in postmenopausal women [23]. However, the effects of different training modalities on S-Klotho in sedentary middle-aged adults of both sexes remain unclear.

Therefore, this study aimed to investigate the effects of different training modalities on the S-Klotho in sedentary middle-aged adults.

MATERIAL & METHODS

Participants

A total of 74 middle-aged adults (45-65 years old; 52.7% women) were enrolled in the FIT-AGEING study, an exercise-based randomised controlled trial (clinicaltrial.gov: ID: NCT03334357) [24]. The participants were required to be sedentary (less than 20 minutes of moderate-intensity physical activity on 3 days/week over the last three months) and to have had a stable weight over the previous 6 months. The participants were recruited in the province of Granada (Spain) using social networks, local media, and posters. The interested individuals were screened via telephone or e-mail. The exclusion criteria included having history of cardiovascular disease, diabetes mellitus, pregnant or lactating women, beta-blockers, and/or major illness (acute or chronic) including any that would limit the ability to perform the necessary exercises. The study procedures were approved by the Human Research Ethics Committee of the "Junta de Andalucía" [0838-N-2017], and the participants provided written informed consent. The study protocols were applied in accordance with the revised ethical guidelines of the Declaration of Helsinki. All of the baseline and follow-up examinations were performed at the same setting [*Centro de Investigación Deporte y Salud (CIDS)* at the University of Granada].

Study design

We conducted a 12-week randomised controlled trial with a parallel group design following the CONSORT (Consolidated Standards of Reporting Trials) guidelines [25]. After completing the baseline measurements, the participants were randomised into 4 different groups using a computer-generated simple randomisation software [26]: (i) control group (no exercise), (ii) a PAR group, (iii) a HIIT group, and (iv) a HIIT+EMS group. The staff in charge of the assessment were blinded to the participants' randomisation. All participants were requested not to alter their eating and physical activity habits during the study, except for those in the exercise groups, who were instructed to do additional exercise as per their intervention programmes.

Training modalities

A detailed description of each training modality can be found elsewhere [24].

PAR underwent a concurrent training (combining aerobic and resistance training) based on the minimum physical activity recommended by the World Health Organization [27]. The participants exercised 3 days/week for 12 weeks. The training volume was 150 min/week at 60-65% of the HRres for the aerobic training. The resistance training volume was ~60 min/week, and the resistance training intensity was set at 40-50% of 1RM. The exercises programmed for the aerobic training section were treadmill, cycle-ergometer, and elliptical ergometer. For the resistance training section, weight bearing and guided pneumatic machines were used (i.e. squat, bench press, dead lift, or lateral pull down). In addition, compensatory exercises were performed (core stability, flexibility, and stabilizer muscles) to minimize risk of injuries and to promote training.

HIIT did an intervention programme characterised by short and intermittent efforts of vigorous activity, interspersed with resting periods at passive or low-intensity exercises. The participants exercised 2 days/week for 12 weeks following 2 different complementary protocols alternatively [28]: (i) HIIT with long intervals (type A session), and (ii) HIIT with short intervals (type B session). The training volume was 40-65 min/week at >95% of the VO_2max in type A session and >120% of the VO_2max in type B session. The exercise programmed for type A session was treadmill with a personalised slope, and type B session included a circuit workout with 8 weight-bearing exercises (i.e. squat, dead lift, high knees up, high heels up, push up, horizontal row, lateral plank, and frontal plank).

The WB-EMS technology enables the simultaneous exogenous muscle activation of up to 18 regions with a total area of 2800 cm^2 covered by electrodes, emerging as an innovative training modality. The HIIT+EMS group completed a training programme that followed the same structure as HIIT (volume, intensity, training frequency, type of exercise, and training sessions). However, we included electrical impulses to assess whether this training modality produced an extra effect in addition to the HIIT protocol. A bipolar, symmetrical, and rectangular electric pulse was applied with: (i) a frequency of 15-20 hertz in type A sessions, and 35-75 hertz in type B sessions, (ii) an intensity of 100 milliamps in type A sessions, and 80 milliamps in type B sessions, (iii) an impulse breadth of 200-400 µsec, and (iv) a duty cycle (ratio of on-time to the total cycle time: % duty cycle = 100/ [total time/on-time]) of 99% in type A sessions and 50-63% in type B sessions. A WB-EMS device manufactured by Wiemspro® (Malaga, Spain) was used.

All sessions started with a dynamic standardised warm-up, which included general mobility exercises, and they ended with a cooling-down protocol (active global stretching), alternating 5 posterior chain exercises with 5 anterior chain exercises. We also proposed a gradual progression to control the exercise dose in each exercise modality [24].

Measurement of S-Klotho

We collected the blood samples from the antecubital vein applying standard techniques at the baseline and after the intervention study.

The S-Klotho was measured in EDTA plasma using a solid-phase sandwich ELISA (Demeditec, Kiel, Germany) according to the manufacturer´s protocol. The intra- and inter-assay coefficients of variation were calculated by measuring two different doses with purified S-Klotho protein. Both coefficients of variation ranged from ~3% to ~10%. All participants were asked to abstain from drugs, alcohol, and/or caffeine, to eat a standardised dinner, and to avoid any physical activity of moderate intensity (24 hours before) and/or vigorous intensity (48 hours before).

Anthropometric and body composition measures

Anthropometric measurements were taken before and after the intervention programme, and we then calculated the BMI (weight/height^2). The weight and height were measured using an electronic scale (model 799, Electronic Column Scale, Hamburg, Germany). LM and FM were evaluated by dual-energy X-ray absorptiometry (Discovery Wi, Hologic, Inc., Bedford, MA, USA) following the manufacturer's recommendations. We also calculated 2 indices of height-normalised body composition: LMI, calculated as LM/height^2, and FMI, calculated as FM/height^2. BMI, LMI, and FMI were expressed in kg/m^2.

Dietary parameters

Dietary intake was registered before and after the intervention program by the average of three 24-hours recalls collected on non-consecutive days (one weekend day included). To quantity the amount of food consumed, we used a coloured photographs of different portion sizes of foods. The EvalFINUT ® software, which is based on USDA (U.S. Department of Agriculture) and BEDCA ("Base de Datos Española de Composición de Alimentos") databases, was used to determine energy intake and macronutrient content derived from the three 24-hours recalls.

Statistical analysis

We based the sample size calculations on a minimum predicted change in S-Klotho of 150 pg/ml between the intervention groups and the control group, and an SD for this change of 150 pg/ml, based on a pilot study. A sample size of 17 participants was predicted to provide a statistical power of 80% considering a type I error of 0.05 [29]. Assuming a maximum loss of 25% at follow-up, we decided to recruit at least 20 participants for each group.

We used the Shapiro-Wilk test, visual check of histograms, and Q-Q plots to verify the distribution of all variables. The descriptive parameters are reported as mean and SD.

We used repeated-measures ANOVA to determine changes in S-Klotho, BMI, LMI, FM percentage, and FMI across time, between groups, and the interaction (time*group). Student's t tests for paired values were performed to evaluate differences in dependent variables before and after the intervention programme.

We examined with the ANCOVA the effect of the groups (fixed factor) on the S-Klotho changes, i.e. post-S-Klotho minus pre-S-Klotho (dependent variable), adjusting for the baseline values. The same analyses were conducted for changes in BMI, LMI, FM percentage, FMI, energy intake, and macronutrient content.

All group-related changes were adjusted by age and sex. We performed Bonferroni post hoc tests with adjustment for multiple comparisons to determine differences between all exercise modality groups.

To examine the relationship between changes in body composition variables (BMI, LMI, FM percentage, and FMI) and changes in S-Klotho, we conducted a multiple linear regression adjusting by sex and age. P values of less than 0.05 were accepted to indicate statistical significance. All analyses were performed using the Statistical Package for Social Sciences (SPSS, v. 22.0, IBM Corporation, Chicago, IL, USA). The graphical presentations were prepared using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

RESULTS

Between April 2015 and December 2016, 141 people were screened. Figure 1 shows the flow of participants from the recruitment to the follow-up. We recruited 102 (41%) of 247 individuals that expressed interest to participate in our study. The most common reasons for refusal were lacking interest (~64%) and being too busy (~27%).

The principal causes to medical exclusion were age or BMI out of range (~28%), having a history of cardiovascular disease (~11%), diabetes mellitus type 2 (~4%), hyper-hypothyroidism (~9%), and abnormal exercise electrocardiogram (~7%). Table 1 shows the participants' baseline characteristics. The groups were similar in age, sex, BMI, LMI, FM percentage, and FMI. From the baseline to week 12, PAR, HIIT, and HIIT-EMS participants attended 98.9% (605 of 612 sessions), 97.8% (399 of 408 sessions), and 99.3% (453 of 456 sessions) of their supervised exercise sessions, respectively. A total of 21 people withdrew (23.6%) between the randomisation and the follow-up: 7 (31.8%) control group participants, 4 (19.4%) PAR participants, 6 (26.1%) HIIT participants, and 4 (17.4%) HIIT-EMS participants. Three people from the control group withdrew for medical reasons, two from the same group withdrew because they were dissatisfied with the randomisation, and the remaining individuals who withdrew from the control and exercise groups due to not having time.

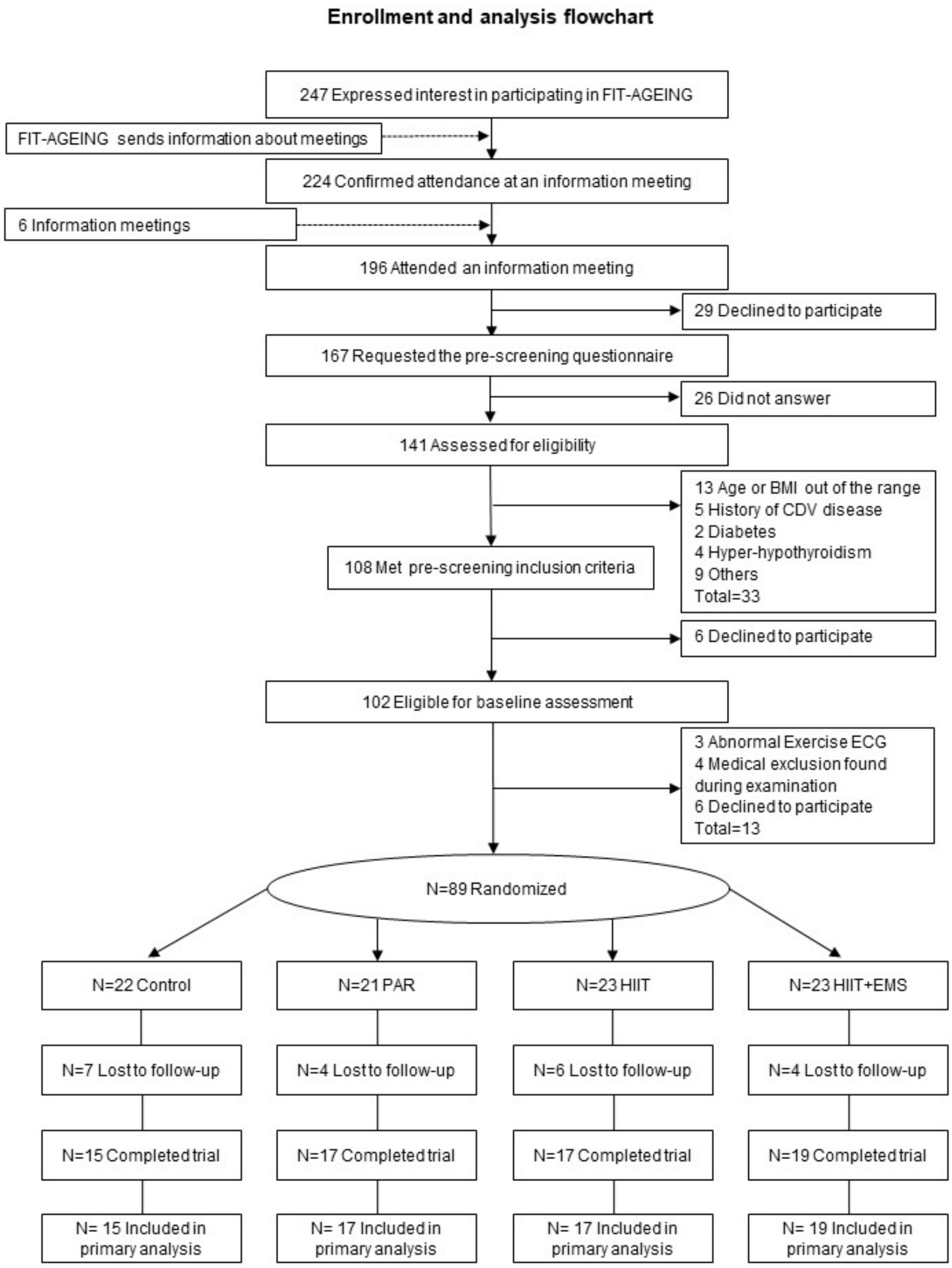

Figure 1. Enrolment and analysis flow-chart. Abbreviations: BMI; body mass index, CDV; cardiovascular, ECG; electrocardiogram, PAR; Physical Activity Recommendations for adults proposed by the World Health Organization group, HIIT; High Intensity Interval Training group, HIIT+EMS; HIIT plus Whole-Body Electromyostimulation group.

Figure 2A shows S-Klotho (Figure 2A) before and after the intervention study. The time*group interaction in S-Klotho was noted ($P<0.001$). When comparing within-group changes, all training modalities showed significantly higher S-Klotho after the intervention programme compared to the baseline (714.3 ± 294.5 vs. 1055.4 ± 435.9 pg/ml for PAR, 788.5 ± 276.8 vs. 1057.1 ± 273.3 pg/ml for HIIT, and 808.5 ± 499.0 vs. 1259.7 ± 613.1 pg/ml for HIIT-EMS; all $P<0.001$). However, we found no differences in the control group (922.5 ± 290.3 vs. 862.9 ± 364.7 pg/ml; $P=0.142$). Figure 2B shows changes in S-Klotho after the intervention study among the 4 groups. Compared with the control group, S-Klotho increased in PAR, HIIT, and HIIT-EMS ($P=0.003$, $P=0.019$, $P<0.001$, respectively) without statistical differences between them (all $P\geq0.696$; Figure 2B). The results persisted after including sex and age in the model (all $P\geq0.170$).

Figure 3 shows the association between changes in body composition variables and changes in S-Klotho after the intervention programmes. Changes in BMI were not significantly associated with those in S-Klotho ($\beta=-41.351$, $R^2=0.007$, $P=0.502$; Figure 3A). A significantly positive association was found between the changes in LMI and in S-Klotho ($\beta=50.119$, $R^2=0.113$, $P=0.008$; Figure 3B), which persisted after including sex and age in the model ($\beta=45.104$, $R^2=0.136$, $P=0.035$). We observed a strong and negative association between the changes in FM outcomes (FM percentage and FMI) and in S-Klotho ($\beta=-16.111$, $R^2=0.136$, $P=0.003$, and $\beta=-51.616$, $R^2=0.138$, $P=0.013$, respectively), which remained unchanged after including sex and age as covariates ($\beta=-14.998$, $R^2=0.162$, $P=0.016$, and $\beta=-47.420$, $R^2=0.164$, $P=0.015$, respectively).

We did not find significant differences in energy intake and macronutrient content after the intervention program in any group ($P\geq0.3$).

Table 1. Descriptive baseline parameters.

	All (n=68)	Control (n=15)	PAR (n=17)	HIIT (n=17)	HIIT+EMS (n=19)
Age (years)	53.4 ± 5.0	51.7 ± 4.1	54.9 ± 4.5	53.5 ± 5.6	53.5 ± 5.2
Sex (%)					
Men	32 (47.1)	6 (40.0)	8 (47.1)	8 (47.1)	10 (52.6)
Women	36 (52.9)	9 (60.0)	9 (52.9)	9 (52.9)	9 (47.4)
BMI (kg/m^2)	26.8 ± 3.9	26.7 ± 3.9	25.4 ± 2.9	26.4 ± 3.2	28.6 ± 4.6
LMI (kg/m^2)	15.4 ± 2.8	15.9 ± 3.1	15.2 ± 2.5	14.6 ± 2.7	15.8 ± 2.9
FM (%)	39.6 ± 8.5	37.7 ± 8.2	37.4 ± 8.8	41.6 ± 8.1	41.3 ± 8.8
FMI (kg/m^2)	10.7 ± 3.1	10.1 ± 2.7	9.6 ± 2.7	11.0 ± 2.6	12.0 ± 3.8
S-Klotho (pg/ml)	805.1 ± 358.8	922.5 ± 290.3	714.3 ± 294.5	788.5 ± 276.8	808.5 ± 499.0

Data are shown as means ± SD. Abbreviations: PAR; Physical Activity Recommendations for adults proposed by the World Health Organization group, HIIT; High Intensity Interval Training group, WB+EMS; HIIT plus Whole-Body Electromyostimulation group.

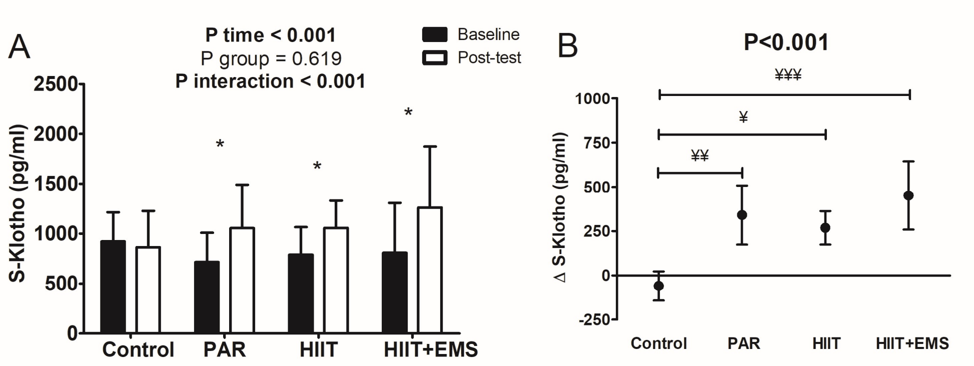

Figure 2. S-Klotho before and after the intervention study. P value (time, group, and interaction [time*group]) of repeated measures ANOVA (Panels A, C, and E). *P<0.05, Student's paired t-test (Panels A, C, and E). Changes in S-Klotho after the intervention study in the 4 groups. ¥ P<0.05, ¥ ¥ P<0.01, ¥ ¥ ¥ P<0.001, ANCOVA adjusting for baseline values, with post hoc Bonferroni-corrected t-test (Panels B, D, and F). The data are shown as means ± standard deviation. Abbreviations: PAR; Physical Activity Recommendations for adults proposed by the World Health Organization group, HIIT; High Intensity Interval Training group, WB+EMS; HIIT plus Whole-Body Electromyostimulation group.

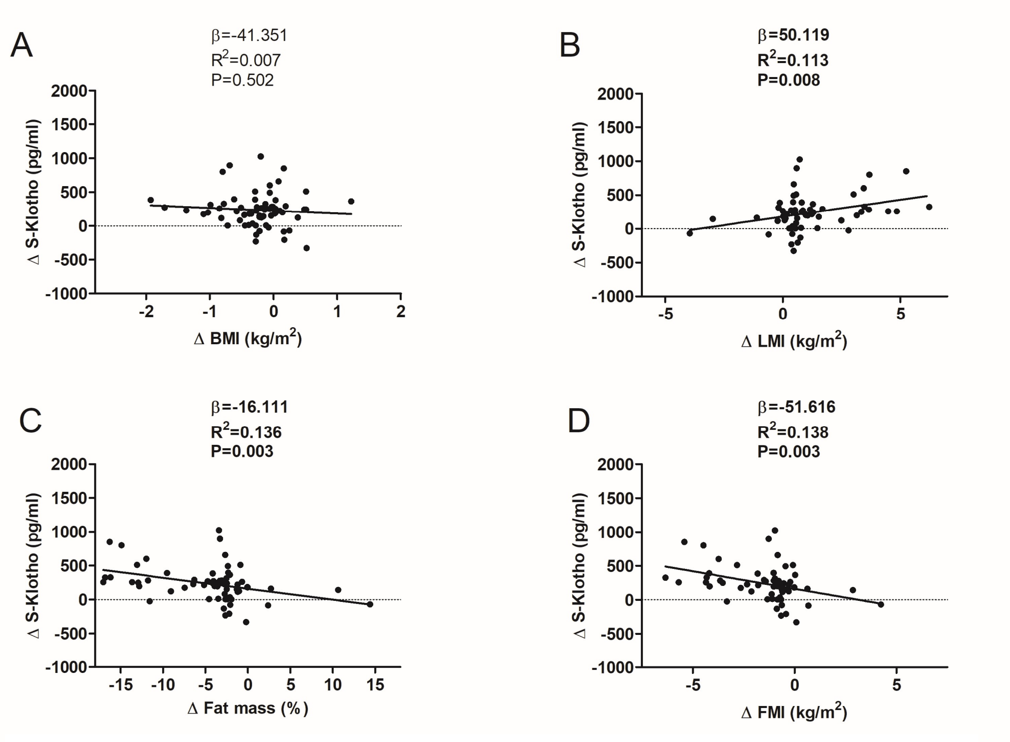

Figure 3. Association between changes in body composition variables which include body mass index (BMI, Panel A), lean mass index (LMI, Panel B), fat mass percentage (Panel C), and fat mass index (FMI, Panel D) with S-Klotho changes in sedentary middle-aged adults. β (unstandardized regression coefficient), R^2, and P from a simple linear regression analysis.

DISCUSSION

The primary finding of this randomised controlled trial is that exercise training induced an increase on S-Klotho in sedentary middle-aged adults. We found a positive association between the changes in LMI and in S-Klotho, whereas a negative association was reported between the changes in FM outcomes (FM percentage and FMI) and in S-Klotho after our intervention study.

S-Klotho mediates the cellular apoptosis and senescence through the alteration of metabolic pathways, which can also be involved in the development of metabolic diseases [30]. It has been shown that inflammation and the oxidative stress induce metabolic impairments [31]; however, S-Klotho suppress the activity of inflammation factors [32] and oxidative stress [33,34], acting as a humoral agent that modulates numerous growth factors and cytokines, which have a close relationship with cell senescence, metabolic disorders, and the ageing process [10,34].

It is well-known that exercise training exerts a powerful anti-ageing effect through two principal mechanisms. On the one hand, although acute intense exercise increases reactive oxygen species and inflammation onset [35], chronic exercise promotes an anti-inflammatory environment (by the reduction of VAT, increasing the production and release of anti-inflammatory cytokines from contracting the skeletal muscle, and by reducing the expression of toll-like receptors on monocytes and macrophages) [36]. On the other hand, exercise training increases the antioxidant cellular capacity (increasing the expressions and activities of key antioxidant enzymes, and inducing the mobilization of non-enzymatic antioxidants to mitochondrial membranes to prevent their oxidative damage) [37,38]. Therefore, an exercise programme that elevates S-Klotho may modulate the production of insulin and IGF-I (which reduce the reactive oxygen species production) [14,34], and the production of TGF-β, Wnt signalling, and IFNγ (which attenuate the inflammatory cell response) [7,10,14]. Hence, it is plausible that an increase in S-Klotho could partially explain the anti-ageing effects produced by exercise training.

However, the relationship between S-Klotho and exercise remains unclear. Avin et al. suggested that S-Klotho is upregulated after a single acute exercise in young and older sedentary women, but that the response may depend on physical fitness levels and age [5]. These findings concur with those of other studies such as Sagiv et al., who studied the effects of an acute exercise consisting in 60 min of treadmill running at 75% of VO_2max on S-Klotho changes in aerobic trained sportsmen compared with anaerobic sprinters [20]. Santos-Diaz et al. showed similar results, revealing that running during only 20 minutes at high intensity increased S-Klotho in healthy young adults [21].

Little is known about the chronic effects of exercise on S-Klotho. Matsubara et al. reported that 12 weeks of moderate aerobic exercise training showed an increase in S-Klotho in 19 healthy postmenopausal women compared with a control group [23]. However, it is unknown whether S-Klotho is influenced by different exercise modalities in sedentary middle-aged adults of both sexes. In the present study, all training modalities induced an increase on S-Klotho in sedentary middle-aged adults compared with a control group. Although no statistical differences in S-Klotho were found between different training modalities, we must consider that S-Klotho changes in HIIT-EMS were higher (55.8%) than in PAR (47.7%) and HIIT (34.1%), which might be of clinical relevance. However, since we did not find significant differences across all exercise group, these differences could be product of a simple variation. Consequently, a larger study is needed to clarify these findings.

Furthermore, certain physiological mechanisms have been proposed as an explanation of how exercise training can modulate S-Klotho. It has been shown that the PPAR-γ, which is related to the cellular inflammation process, the adipogenesis process, and glucose homeostasis, increases the α-Klotho gene expression in the kidney [39]. On the contrary, angiotensin II downregulates the α-Klotho gene expression in the kidney [40], and blockade of the angiotensin II type I receptor results in an over-expression of the α-Klotho gene [41]. The reactive oxygen species also decrease the α-Klotho gene expression in mice kidney cells [42], and it is suggested that a free radical scavenger can upregulate the α-Klotho gene expression [43]. Interestingly, several studies have found that exercise training increased the activity of PPAR-γ [44] and decreased the angiotensin II type I receptors [45] and oxidative stress [37,38]. Thus, exercise training could increase the α-Klotho gene expression (and consequently, S-Klotho) through an upregulation in the PPAR-γ activity, and a downregulation of the angiotensin II type I receptors and oxidative stress in the kidney.

It is well-known that LM tends to decline with age, accompanied by a relative increase of FM even when the weight remains stable [46]. Our results showed a positive association between the changes in LMI in S-Klotho, and a negative association between the changes in FM outcomes (FM percentage and FMI) and in S-Klotho after an exercise intervention. These findings suggest that changes in S-Klotho could be strongly influenced by changes in body composition during an exercise intervention. Several physiological mechanisms could be involved in these relationships. On the one hand, age-related muscular atrophy, characterised by a decreased stem cell quantity, an alteration of the angiogenesis process, and a lower cellular resistance to stress, could produce a defect in the α-Klotho gene expression [5,34,47]. However, an increase in S-Klotho induced by exercise training inhibits the TGF-β and the Wnt signalling (which are considered the "master switch" for promoting mesenchymal transition toward a fibroblastic lineage in several tissues), producing a LM development [5,7,14,48], which left the association's direction indeterminate. On the other hand, age-related adiposity, characterised by a 5-25% decrease in resting metabolic rate, high amount of time spent in sedentary behaviours, or glucose metabolism disorders, could induce a downregulation of the α-Klotho gene expression [49]. The upregulation of the α-Klotho gene induced by exercise training could be associated with a glucose metabolism [50] and phosphate [9] metabolism regulation, and, consequently with reduced type 2 diabetes mellitus risk [51].

Limitations

The present study had a number of limitations. Due to the limited sample size, our study may have been underpowered to detect statistical differences in S-Klotho between the different training modalities, although we did find an increment of S-Klotho in PAR, HIIT, and HIIT-EMS compared with the control group. Our study only included sedentary middle-aged adults (45-65 years old), and hence we cannot extend the results to older, younger, and/or physically active individuals. We did not measure the intra-cellular form and the cell-membrane form of the α-Klotho gene, which would have allowed to better understand the role of exercise on the α-Klotho gene regulation. Finally, we did not measure the insulin, IGF-I, TGF-β, Wnt signalling, and IFNγ, so we cannot know whether S-Klotho changes induced by chronic exercise could be mediated by these parameters.

CONCLUSION

In conclusion, our results show that exercise training induced an increase in S-Klotho in sedentary middle-aged adults and an association between changes in body composition and in S-Klotho after an exercise training programme. Therefore, we suggest that the link between exercise training and the increase in S-Klotho could be mediated by a body re-composition, through a decrease of FM and an increase of LM. Future studies are needed to clarify the long-term effects (>12 weeks) of different training volumes and intensities on S-Klotho as well as on the intracellular form and the cell-membrane form of the α-Klotho gene in sedentary middle-aged adults. Subsequently, further studies are required to determine the effects of the same training interventions in older, younger, physically active individuals, and/or patients to identify effective public health strategies that promote a healthy ageing lifestyle and to reduce the prevalence of chronic diseases associated with the ageing process.

REFERENCES

1 Darren W, Crystal-Whitney N, Shannon B. Health benefits of physical activity: the evidence. *Can Med Assoc J* 2006; **174**: 801–809.

2 Pedersen B, Saltin B. Exercise as medicine - Evidence for prescribing exercise as therapy in 26 different chronic diseases. *Scand J Med Sci Sport* 2015; **25**: 1–72.

3 Gremeaux V, Gayda M, Lepers R, Sosner P, Juneau M, Nigam A. Exercise and longevity. *Maturitas* 2012; **73**: 312–317.

4 Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T *et al.* Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* 1997; **390**: 45–51.

5 Avin K, Coen P, Huang W, Stolz D, Sowa G, Dubé J *et al.* Skeletal muscle as a regulator of the longevity protein, Klotho. *Front Physiol* 2014; **5**: 1–9.

6 Kurosu H, Yamamoto M, Clark JD, Pastor J V, Nandi A, Gurnani P *et al.* Suppression of aging in mice by the hormone Klotho. *Science* 2005; **309**: 1829–33.

7 Liu H, Fergusson MM, Castilho RM, Liu J, Cao L, Chen J *et al.* Augmented Wnt signaling in a mammalian model of accelerated aging. *Science* 2007; **317**: 803–6.

8 Rubinek T, Wolf I. The role of Alpha-Klotho as a universal tumor suppressor. *Vitam Horm* 2016; **101**: 197–214.

9 Hu MC, Shiizaki K, Kuro-o M, Moe OW. Fibroblast Growth Factor 23 and Klotho: Physiology and pathophysiology of an endocrine network of mineral metabolism. *Annu Rev Physiol* 2013; **75**: 503–533.

10 Kim J-H, Hwang K-H, Park K-S, Kong ID, Cha S-K. Biological role of anti-aging protein Klotho. *J lifestyle Med* 2015; **5**: 1–6.

11 Chang Q, Hoefs S, van der Kemp AW, Topala CN, Bindels RJ, Hoenderop JG. The beta-glucuronidase klotho hydrolyzes and activates the TRPV5 channel. *Science* 2005; **310**: 490–3.

12 Bloch L, Sineshchekova O, Reichenbach D, Reiss K, Saftig P, Kuro-o M *et al.* Klotho is a substrate for alpha-, beta- and gamma-secretase. *FEBS Lett* 2009; **583**: 3221–4.

13 Imura A, Iwano A, Tohyama O, Tsuji Y. Secreted Klotho protein in sera and CSF: implication for post-translational cleavage in release of Klotho protein from cell membrane. *FEBS Lett* 2004; **565**: 143–147.

14 Doi S, Zou Y, Togao O, Pastor J V., John GB, Wang L *et al.* Klotho inhibits Transforming Growth Factor-β1 (TGF-β1) signaling and suppresses renal fibrosis and cancer metastasis in mice. *J Biol Chem* 2011; **286**: 8655–8665.

15 Sakan H, Nakatani K, Asai O, Imura A, Tanaka T, Yoshimoto S *et al.* Reduced renal α-Klotho expression in CKD patients and its effect on renal phosphate handling and vitamin D metabolism. *PLoS One* 2014; **9**: e86301.

16 Semba R, Cappola A, Sun K. Plasma klotho and cardiovascular disease in adults. *J Am Geriatr Soc* 2011; **59**: 1596–1601.

17 Otani-Takei N, Masuda T, Akimoto T, Honma S, Watanabe Y, Shiizaki K *et al.* Association between serum soluble Klotho levels and mortality in chronic hemodialysis patients. *Int J Endocrinol* 2015; **2015**: 1–10.

18 Amaro-Gahete FJ, de la O A, Jurado-Fasoli L, Ruiz JR, Castillo MJ, Gutierrez A. Role of exercise on S-Klotho protein regulation: a systematic review. *Curr Aging Sci* 2018; **11**.

19 Saghiv M, Goldhammer E, Sagiv M, Ben-Sira D. Effects of aerobic exercise training on S-Klotho in young and elderly. *J J Physiol* 2015; **1**: 1–2.

20 Saghiv M, Sagiv M, Ben-Sira D. Comparison between aerobic and anaerobic training influence on s-klotho blood levels following 60 min aerobic bout. *J J Physiol* 2015; **1**: 1–4.

21 Santos-Dias A, MacKenzie B, Oliveira-Junior MC, Moyses RM, Consolim-Colombo FM, Vieira RP. Longevity protein klotho is induced by a single bout of exercise. *Br J Sports Med* 2017; **51**: 549–550.

22 Mostafidi, E, Moeen, A, Nasri, H, Ghorbani-Hagjo, A, Ardalan M. Serum Klotho levels in trained athletes. *Nephrourol Mon* 2016; **8**: 12–17.

23 Matsubara T, Miyaki A, Akazawa N, Choi Y, Ra S-G, Tanahashi K *et al.* Aerobic exercise training increases plasma Klotho levels and reduces arterial stiffness in postmenopausal women. *Am J Physiol Heart Circ Physiol* 2014; **306**: H348-55.

24 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.

25 Schulz KF, Altman DG, Moher D, Jüni P, Altman D, Egger M *et al.* CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMC Med* 2010; **8**: 18.

26 Schulz KF, Grimes DA. Generation of allocation sequences in randomised trials: chance, not choice. *Lancet (London, England)* 2002; **359**: 515–9.

27 WHO. Global recommendations on physical activity for health, World Health Organ publications. Geneva, Switzerland, 2015.

28 Buchheit M, Laursen PB. High-intensity interval training, solutions to the programming puzzle: Part I: Cardiopulmonary emphasis. *Sport Med* 2013; **43**: 313–338.

29 Shaffer ML, Chinchilli VM. Including multiple imputation in a sensitivity analysis for clinical trials with treatment failures. *Contemp Clin Trials* 2007; **28**: 130–137.

30 Razzaque MS. The role of Klotho in energy metabolism. *Nat Rev Endocrinol* 2012; **8**: 579–587.

31 Hotamisligil GS. Inflammation and metabolic disorders. *Nature* 2006; **444**:

860–867.

32 Liu S, Wu S, Ren H, Liu, S, Wu, S, Ren, H, Gu J. Klotho suppresses RIG-I-mediated senescence-associated inflammation. *Nat Cell Biol* 2011; **13**: 254–262.

33 Kuro-o M. Klotho as a regulator of oxidative stress and senescence. *Biol Chem* 2008; **389**: 233–241.

34 Yamamoto M, Clark JJD, Pastor J V., Gurnani P, Nandi A, Kurosu H *et al.* Regulation of oxidative stress by the anti-aging hormone klotho. *Biol Chem* 2005; **280**: 3829–3824.

35 Pillon Barcelos R, Freire Royes LF, Gonzalez-Gallego J, Bresciani G. Oxidative stress and inflammation: liver responses and adaptations to acute and regular exercise. *Free Radic Res* 2017; **51**: 222–236.

36 Gleeson M, Bishop NC, Stensel DJ, Lindley MR, Mastana SS, Nimmo MA. The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. *Nat Rev Immunol* 2011; **11**: 607–15.

37 Bouzid MA, Filaire E, McCall A, Fabre C. Radical oxygen species, exercise and aging: an update. *Sport Med* 2015; **45**: 1245–1261.

38 Huertas JR, Al Fazazi S, Hidalgo-Gutierrez A, López LC, Casuso RA. Antioxidant effect of exercise: Exploring the role of the mitochondrial complex I superassembly. *Redox Biol* 2017; **13**: 477–481.

39 Zhang H, Li Y, Fan Y, Wu J, Zhao B, Guan Y *et al.* Klotho is a target gene of PPAR-γ. *Kidney Int* 2008; **74**: 732–739.

40 Mitani H, Ishizaka N, Aizawa T, Ohno M, Usui S, Suzuki T *et al.* In vivo klotho gene transfer ameliorates angiotensin II-induced renal damage. *Hypertension* 2002; **39**: 838–43.

41 Yu L, Meng W, Ding J, Cheng M. Klotho inhibits angiotensin II-induced cardiomyocyte hypertrophy through suppression of the AT1R/beta catenin pathway. *Biochem Biophys Res Commun* 2016; **473**: 455–61.

42 Mitobe M, Yoshida T, Sugiura H, Shirota S, Tsuchiya K, Nihei H. Oxidative stress decreases klotho expression in a mouse kidney cell line. *Nephron Exp Nephrol* 2005; **101**: e67-74.

43 Saito K, Ishizaka N, Mitani H, Ohno M, Nagai R. Iron chelation and a free radical scavenger suppress angiotensin II-induced downregulation of klotho, an anti-aging gene, in rat. *FEBS Lett* 2003; **551**: 58–62.

44 Tomeleri CM, Souza MF, Burini RC, Cavaglieri CR, Ribeiro AS, Antunes M *et al.* Resistance training reduces metabolic syndrome and inflammatory markers in older women: A randomized controlled trial. *J Diabetes* 2018; **10**: 328–337.

45 Muñoz A, Corrêa CL, Lopez-Lopez A, Costa-Besada MA, Diaz-Ruiz C, Labandeira-Garcia JL. Physical exercise improves aging-related changes in angiotensin, IGF-1, SIRT 1, SIRT3 and VEGF in the substantia nigra. *journals*

Gerontol Ser A, Biol Sci Med Sci 2018; In press.

46 Molino S, Dossena M, Buonocore D, Verri M. Sarcopenic obesity: an appraisal of the current status of knowledge and management in elderly people. *J Nutr Health Aging* 2016; **20**: 780–8.

47 Izbeki F, Asuzu DT, Lorincz A, Bardsley MR, Popko LN, Choi KM *et al.* Loss of Kit low progenitors , reduced stem cell factor and high oxidative stress underlie gastric dysfunction in progeric mice. 2010; **16**: 3101–3117.

48 Zhou L, Li Y, Zhou D, Tan RJ, Liu Y. Loss of Klotho contributes to kidney injury by depression of Wnt/catenin signaling. *J Am Soc Nephrol* 2013; **24**: 771–785.

49 JafariNasabian P, Inglis JE, Reilly W, Kelly OJ, Ilich JZ. Aging human body: changes in bone, muscle and body fat with consequent changes in nutrient intake. *J Endocrinol* 2017; **234**: R37–R51.

50 Donate-Correa J, Martín-Núñez E, Delgado NP, de Fuentes MM, Arduan AO, Mora-Fernández C *et al.* Implications of Fibroblast growth factor/Klotho system in glucose metabolism and diabetes. *Cytokine Growth Factor Rev* 2016; **28**: 71–77.

51 Nie F, Wu D, Du H, Yang X, Yang M, Pang X *et al.* Serum klotho protein levels and their correlations with the progression of type 2 diabetes mellitus. *J Diabetes Complications* 2017; **31**: 594–598

Chapter 10:

Effects of different exercise training programs on body composition: A randomized control trial
(Study 14)

ABSTRACT

This study aimed to investigate the effects of different exercise training programs on body composition parameters in sedentary middle-aged adults.

A total of 89 middle-aged adults (53.5±4.9 years old; ~53% women) participated in the FIT-AGEING study. A 12-week randomised controlled trial was performed with a parallel group design. The participants were randomly assigned to: [i] a PAR group, [ii] a HIIT group, and [iii] a HIIT+EMS group.

A significant decrease of FM, FMI and VAT were observed in all training modalities compared to the control group (all P≤0.001). There was a significant increase in LM in the HIIT group as well as in the HIIT+EMS group compared to the control group and the PAR group (all P≤0.044), whereas an increment of LMI was only observed in the HIIT+EMS group compared to the control group and the PAR group (all P≤0.042). A significant increase of bone mineral content was observed in the HIIT+EMS group compared to the control group (P=0.015), while no changes were found in the PAR group and in the HIIT group compared to the control group (all P≥0.2).

Our findings suggest that PAR, HIIT and HIIT+EMS can be used as a strategy to improve body composition parameters, obtaining slightly better results with the application of HIIT+EMS.

BACKGROUND

It is well-known that exercise training provides important benefits on body composition [1]. Concurrent training (which combines resistance and endurance training) induced a decrease of FM and an increment of LM in both sedentary men [2] and women [3]. Moreover, it has been previously reported that a resistance training program improved BMD in sedentary men [4] and women [5]. However, considering that time constraints have been usually reported as the main limitation to follow an exercise program [6], HIIT seems to be a feasible time-efficient strategy to improve body composition [7]. A recent systematic review observed that moderate intensity continuous exercise programs and HIIT programs result in similar improvements in FM in overweight and obese sedentary individuals, thus, considering that HIIT programs imply 40% less time commitment each week, the authors concluded that HIIT can be considered a time-efficient alternative for managing overweight and obese individuals [7]. In addition, Nybo et al. showed that LM and BMC remained unchanged after a HIIT program, whereas significant improvements in LM and BMC were obtained after the application of a 12-weeks resistance training program [8]. Taken together, the above findings suggested that positive but inconclusive results have been previously reported about the influence of HIIT on body composition parameters for individuals with different ages and biological characteristics.

Despite HIIT is currently the trendiest time-efficient exercise method, alternative exercise training programs are arising. WB-EMS is able to stimulate all the main muscle groups with dedicated intensity simultaneously [9], and has become increasingly popular during the last decade. A total of 20 studies have investigated its influence on physical fitness and health in trained and untrained individuals, and in patients who cannot perform conventional modalities of exercise because of physical or mental illness [10–29]. Most of these studies (n=10) have examined the role of WB-EMS on body composition parameters [10,11,14,17,19,21,23,26,28,29], showing that this training modality induced a generally decrease of FM and a generally increase of LM in (i) sedentary older men [10,11] and women with sarcopenic obesity [17,29] (all of them aged >70 years old), (ii) in sedentary older women with obesity (aged >70 years old) [19], (iii) in moderately trained male runners [23], (iv) in sedentary healthy men (aged 30 to 50 years old) [28], and (v) in moderately trained healthy women (aged >55 years old) [21]. In addition, a study conducted in sedentary older women with osteopenia (aged >70 years old) suggested that a 54-weeks WB-EMS program could be an option for maintaining BMD [14].

To our knowledge, there is only one study comparing the effects of a HIIT-resistance program vs. a WB-EMS program [28]. The main findings were that both of them were equally effective, attractive, feasible and time-efficient methods for combatting cardio-metabolic risk factors (which included FM, but not LM) in untrained middle-aged men [28]. However, this study does not allow to know if there is an added effect on body composition parameters when WB-EMS is applied together with a HIIT program, since different exercises and training load approaches were performed in each experimental group (HIIT-resistance vs. low-intensity resistance program with WB-EMS). Moreover, there is a lack of studies comparing the influence of different exercise training programs (i.e. concurrent training vs. HIIT vs. HIIT+EMS) on body composition parameters in sedentary middle-aged adults. Therefore, this study aimed to investigate the effects of different exercise training programs ([i] a PAR group, [ii] a HIIT group, and [iii] a HIIT+EMS group) on body composition parameters in sedentary middle-aged adults.

MATERIAL & METHODS

Participants

A total of 89 middle-aged adults (52.7 % women), aged between 45-65 years were enrolled in the FIT-AGEING study, an exercise-based randomized controlled trial (clinicaltrial.gov: ID: NCT03334357) [30]. The study was approved by the Ethics Committee on Human Research at the University of Granada and "Servicio Andaluz de Salud" (CEI-Granada) [0838-N-2017] and all participants signed an informed consent. The study protocols and experimental design were applied in accordance with the last revised ethical guidelines of the Declaration of Helsinki. The participants were recruited from the province of Granada (Spain) using social networks, local media, and posters. Interested individuals were screened via telephone, and/or e-mail. Inclusion criteria were: (i) to be sedentary (<20 minutes of moderate-intensity physical activity on 3 days/week over the last three months); (ii) to have a stable weight over the last 6 months; (iii) to be free of disease, pregnant or lactating women; (iv) not taking any medication and/or (v) to suffer major illness that would limit the capacity to perform all exercise training program. Baseline and follow-up assessment were performed at the same setting [*Centro de Investigación Deporte y Salud (CIDS)* at the University of Granada].

Study design

A 12-week randomized control trial with a parallel group design was conducted following the CONSORT (Consolidated Standards of Reporting Trials) guidelines [31]. After the baseline examination, the participants were randomly assigned into 4 different groups using a computer-generated simple randomization: (i) the control group (no exercise), (ii) the PAR group, (iii) the HIIT group, and (iv) the HIIT+EMS group. The participant's randomization allocation was blinded to the assessment staff. All participants were requested not to modify their dietary and physical activity habits the same as before the study, except for those in the exercise group, who were instructed not to do additional exercise as per their intervention programs.

Exercise training programs

A specific description of each exercise training program can be found elsewhere [30]. Briefly, the PAR group performed a concurrent training based on the minimum physical activity recommended by the World Health Organization [32]. Training frequency was 3 sessions/week for 12 weeks. Training volume was 150 min/week at 60-65% of the HRres for the endurance training. Treadmill, cycle-ergometer, and elliptical ergometer were used to perform the endurance training. Resistance training volume was ~60 min/week, and the intensity was set at 40-50% of 1RM. Weight bearing, and guided pneumatic machines were used to perform the resistance training (i.e. squat, bench press, dead lift, or lateral pull down). The participants did compensatory exercises (core stability, flexibility, and stabilizers muscles) to minimize risk of injuries as well as to encourage training adherence.

The HIIT group performed a high intensity interval program characterized by short and intermittent efforts of vigorous activity, interspersed with resting periods at passive or low-intensity exercises. The participants exercised 2 days/week for 12 weeks following 2 different complementary protocols alternatively [33]: (i) HIIT with long intervals (type A session), and (ii) HIIT with short intervals (type B session). The training volume was 40-65 min/week, and the training intensity was >95% of VO_2max in type A session, and 6-9 of the ratings of perceived exertion scale [34] in type B session. The exercise chosen for type A session was treadmill with a personalized slope, and the exercises programmed for the type B session were 8 weight-bearing exercises in circuit form (i.e. squat, dead lift, high knees up, high heels up, push up, horizontal row, lateral plank, and frontal plank).

The HIIT+EMS group performed a training program that followed the same structure that HIIT in terms of training frequency, training volume, training intensity and type of exercise. However, we included WB-EMS to check whether this training program induces an additional effect on body composition parameters. Bipolar, symmetrical, and rectangular electric pulse was applied with: (i) a frequency of 15-20 hertz in type A sessions, and 35-75 hertz in type B sessions, (ii) an intensity of 100 milliamps in type A sessions, and 80 milliamps in type B sessions, (iii) an impulse breadth of 200-400 µsec (thigh zone=400 µsec, glute zone=350 µsec, abdominal zone=300 µsec, dorsal zone=250 µsec, cervical zone=200 µsec, chest zone=200 µsec, and arm zone=200 µsec), and (iv) a duty cycle (ratio of on-time to the total cycle time: % duty cycle = 100/ [total time/on-time]) of 99% in type A sessions, and 50-63% in type B sessions. A WB-EMS device manufactured by Wiemspro® (Malaga, Spain) was used.

All training sessions started with a dynamic standardized warm-up, that included general mobility exercises, and ended with a cooling-down protocol (active global stretching), which alternated 5 posterior chain exercises with 5 anterior chain exercises. A gradual progression was also proposed to control the exercise dose in each training group [30]. No adverse events were registered in any group.

Body composition assessment

Body composition assessment was performed before and after the intervention program. Weight and height were measured without shoes and with light clothing, using a pre-validated scale and stadiometer (model 799, Electronic Column Scale, Hamburg, Germany) and the BMI was calculated (weight/height^2). WC was assessed at the mid-point between the bottom of the rib cage and the iliac crest at the end of a normal expiration, and HC was measured at the widest point of the hip. WHr was calculated by dividing waist measurement by hip measurement.

A dual-energy X-ray absorptiometry scanner (Discovery Wi, Hologic, Inc., Bedford, MA, USA) was used to measure FM (kg), VAT (g), LM (kg), and BMC (g) following the manufacturer's recommendations. The whole-body scan was used to obtain all body composition parameters. LMI was calculated as LM in kg divided by height in meters^2, FMI as FM in kg divided by height in meters^2, and BMD as BMC in g divided by the total bone surface in centimeters^2. FM was also expressed as percentage of weight.

Physical activity and sedentary time assessment

We measured physical activity levels with a wrist-worn accelerometer (ActiGraph GT3X+, Pensacola, FL, US) during 7 consecutive days (24 hours/day) before and after the intervention program [30]. The participants were cited in the research center, and we gave specific instruction about how to wear the accelerometer, including to remove it only during water activities (bathing or swimming) between others. A sampling frequency of 100 Hz were selected to store raw accelerations [35]. We used the ActiLife v.6.13.3 software (ActiGraph, Pensacola, FL, US) to export and convert raw data to ".csv" format. Then, these files were processed with the GGIR package (v. 1.5-12, https://cran.r-project.org/web/packages/GGIR/) in R (v. 3.1.2, https://www.cran.r-project.org/). Briefly, the processing methods included a local gravity data auto-calibration [36], a Calculation of the Euclidean Norm Minus One, determination of non-wear time based on the raw acceleration of the three axes, identification of abnormal high accelerations that indicate a malfunctioning of the accelerometers, imputation of non-wear time and abnormal high accelerations, determination of waking and sleeping time by an automatized algorithm guided by the participants' diary reports [37] and the calculation of sedentary time, LPA, MPA, VPA and MVPA using age-specific cut-points for Euclidean Norm Minus One [38]. We included in the analysis only participants which wearing the accelerometers for at least 16 hours/day during 4 days (including 1 weekend day).

Dietary intake assessment

Dietary intake was registered before and after the intervention program by the average of three 24-hours recalls collected on non-consecutive days (one weekend day included), which is a valid method to determine energy intake to within 8–10% of the real energy intake [39]. We conducted an interview, in which a detailed description of the food consumed by the participants were recorded. To help estimate the quantity of food consumed, we used a coloured photographs of different portion sizes of foods. The EvalFINUT ® software, which is based on USDA (U.S. Department of Agriculture) and BEDCA ("Base de Datos Española de Composición de Alimentos") databases, was used to determine energy intake and macronutrient content derived from the three 24-hours recalls.

Statistical analysis

Sample size calculations were based on a minimum predicted 15% change in FMI, LMI and BMD between the intervention groups and the control group, with an expected SD of 15%. A sample size of 14 participants was predicted to provide a statistical power of 85% considering a type I error of 0.05, based on a pilot study. However, we recruited a minimum of 20 participants per group (a total of 80) to accommodate for a maximum loss of 25% at follow-up.

Data was assessed for normality to ensure that the assumptions of the analysis were met, using Shapiro-Wilk test, visual check of histograms, and Q-Q plots). The descriptive parameters are reported as mean and SD.

We conducted repeated-measures ANOVA to determine changes in BMI, WC, HC, WHr, FM, FM percentage, FMI, VAT, LM, LMI, BMC, and BMD across time, between groups, and the interaction (time*group). We conducted a Student's t tests for paired values to determine differences in dependent variables before and after the intervention programme. The same analysis was used to determine changes in dietary intake, macronutrient content, sedentary time and physical activity levels.

We conducted ANCOVA to analyse the effect of the groups (fixed factor) on body composition parameters, i.e. post-BMI minus pre-BMI (dependent variable), adjusting for the baseline values. The same analyses were conducted for changes in WC, HC, WHr, FM, FM percentage, FMI, VAT, LM, LMI, BMC and BMD. All group-related changes were adjusted by age, sex, changes in dietary intake, changes in macronutrient content, changes in sedentary time and/or changes in physical activity levels when it was required. To determine changes between all exercise modalities, we applied Bonferroni post hoc tests with adjustment for multiple comparisons.

The level of significance was assumed at $P \leq 0.05$. We used the Statistical Package for Social Sciences (SPSS, v. 22.0, IBM Corporation, Chicago, IL, USA) to perform the statistical analysis. Graphical plots were generated using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

RESULTS

Participant flow-chart is presented in Figure 1. Loss to follow-up was 27% (control group: 36%; PAR: 24%; HIIT: 30%; HIIT+EMS: 17%). The main reasons were that participants reported not having time (control group: n=1; PAR: n=2; HIIT: n=3; HIIT+EMS: n=1), medical reasons (control group: n=3; HIIT: n=1), job related relocation (control group: n=1; PAR: n=1; HIIT: n=1; HIIT+EMS: n=2), or disagreement with the randomization (control group: n=2), while a total of 6 participants (control group: n=1; PAR: n=2; HIIT: n=2; HIIT+EMS: n=1) did not report any reason to leave the study. A total of 65 participants were included in the analysis. Participants attended to 98.9% (605 of 612 sessions), 97.8% (399 of 408 sessions), and 99.3% (453 of 456 sessions) of their supervised exercise sessions in PAR, HIIT and HIIT+EMS, respectively, from baseline to week 12.

Table 1 describes the baseline and post-intervention characteristics of the total groups and of each separate group. There were no significant differences between groups on any baseline characteristic. There were a nearly equal number of men and women in each group.

Figure 2 shows BMI, WC, HC and WHr before and after the intervention study. A significant time*group interaction was found in WC and WHr ($P<0.001$ and $P=0.006$, respectively). BMI decreased in the PAR group as well as in the HIIT+EMS group ($P=0.006$ and $P=0.050$, respectively.

WC decreased in the PAR group as well as in the HIIT group and in the HIIT+EMS group ($P=0.027$, $P<0.001$, and $P<0.001$, respectively). HC decreased in the HIIT group as well as in the HIIT+EMS group ($P=0.008$, and $P=0.005$, respectively). WHr decreased in the HIIT group as well as in the HIIT+EMS group ($P<0.001$, and $P<0.001$, respectively). However, no significant differences were observed in the control group in any case (all $P>0.077$).

A significant time*group interaction was found for FM, FM percentage, FMI and VAT ($P=0.002$, $P=0.004$, $P=0.008$ and $P=0.005$, respectively, Figure 3). FM, FM percentage, FMI and VAT decreased in the PAR group as well as in the HIIT group and in the HIIT+EMS group (all $P\leq0.003$). However, we found no significant differences in the control group in any case (all $P>0.8$).

A significant time*group interaction was found in LM, LMI, BMC and BMD ($P=0.003$,

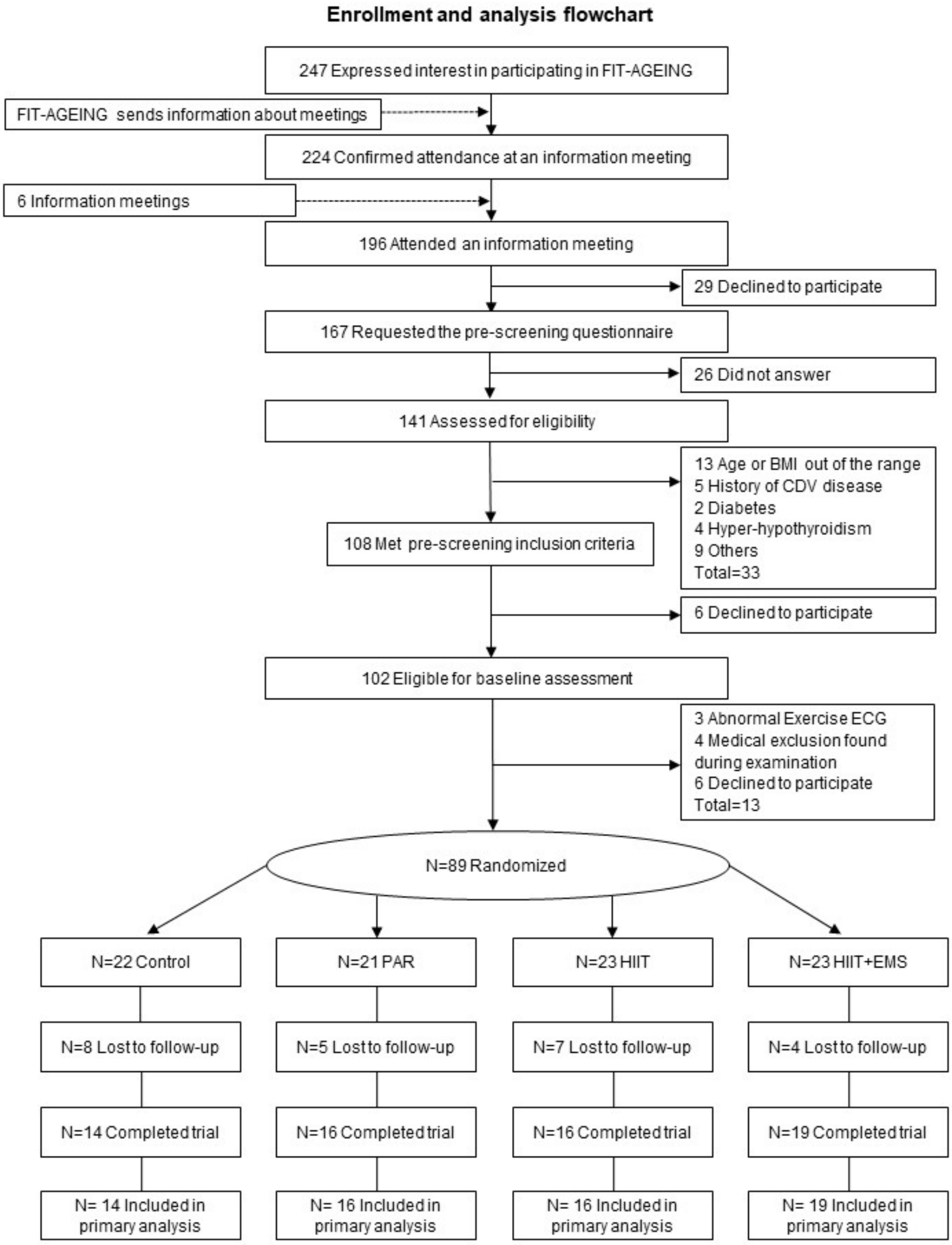

Figure 1. Flow-chart diagram. Abbreviations: BMI; body mass index, CDV; cardiovascular, ECG; electrocardiogram, PAR; Physical Activity Recommendations for adults proposed by the World Health Organization group, HIIT; High Intensity Interval Training group, HIIT+EMS; HIIT plus Whole-Body Electromyostimulation group.

P=0.002, P=0.021 and P=0.002, respectively, Figure 4). LM and LMI increased in the PAR group as well as in the HIIT group and in the HIIT+EMS group (all P≤0.022). BMC increased in the HIIT+EMS group (P=0.043). However, we found no significant differences in the control group in any case (all P>0.1).

Figure 5 shows changes in BMI, WC, HC and WHr after the intervention study among the 4 groups. ANCOVA revealed significant differences between groups in BMI, WC and WHr (P=0.002, P<0.001 and P=0.005, respectively), whereas no significant differences were noted in HC (P=0.621). The results persisted in all cases when sex, age, total physical activity changes and total energy intake changes were included in the model as a covariate (BMI: all P≤0.005, WC: all P≤0.001, HC: all P≥0.6, and WHr: all P≤0.005, see Table 2). Compared with HIIT and HIIT+EMS, BMI decreased in PAR (P=0.011 and P=0.029, respectively). Moreover, a significantly lower WC were noted in HIIT and HIIT+EMS groups compared with the control group (P<0.001 and P=0.002, respectively).

Figure 6 shows changes in FM, FM percentage, FMI and VAT after the intervention study in the 4 groups. ANCOVA revealed significant differences between groups in FM, FM percentage, FMI and VAT (all P<0.001). The results persisted in all cases when sex, age, total physical activity changes and total energy intake changes were included in the model as a covariate (FM: all P<0.001, FM percentage: all P<0.001, FMI: all P<0.001, and VAT: all P<0.001, see Table 2).

DISCUSSION

Here we studied the effects of different exercise training programs on body composition parameters in sedentary middle-aged adults. The primary findings of this randomized control trial were that: (i) A significant decrease of FM, FM percentage, FMI and VAT were observed in the PAR group as well as in the HIIT+EMS group compared to the control group. (ii) There was significant increase in LM in the HIIT group as well as in the HIIT+EMS group compared to the control group, whereas an increment of LMI was only observed in the HIIT+EMS group compared to the control group and the PAR group. (iii) A significant increase of BMC was observed in the HIIT+EMS group compared to the control group, while no changes were found in the PAR group and in the HIIT group compared to the control group.

Table 1. Descriptive parameters

	All (n=65)		Control (n=14)		PAR (n=16)		HIIT (n=16)		HIIT+EMS (n=19)	
Age (years)	53.5 ± 4.9		51.8 ± 4.2		54.6 ± 4.4		53.7 ± 5.7		53.5 ± 5.2	
Sex (%)										
Men	32 (49.2)		6 (42.9)		7 (43.8)		9 (56.3)		10 (52.6)	
Women	33 (50.8)		8 (57.1)		9 (56.3)		7 (43.8)		9 (47.4)	
	PRE	**POST**	**PRE**	**POST**	**PRE**	**POST**	**PRE**	**POST**	**PRE**	**POST**
Body composition parameters										
Weight (kg)	76.4 ± 15.2	75.4±14.7	73.2 ± 13.7	72.7±14.0	71.8 ± 11.1	70.1±9.7	79.1 ± 18.2	78.5±17.3	80.2 ± 15.9	79.3±15.8
Height (cm)	168.3 ± 9.8	168.0±9.8	166.2 ± 8.9	166.2±9.2	168.4 ± 9.5	168.2±9.5	171.2 ± 11.6	170.7±11.4	167.2 ± 9.1	166.8±9.2
Body mass index (kg/m²)	26.8 ± 3.9	26.6±3.8	26.4 ± 3.8	26.2±3.6	25.3 ± 2.9	24.7±2.4	26.6 ± 3.3	26.6±3.1	28.6 ± 4.6	28.4±4.6
Waist circumference (cm)	95.1± 12.2	92.2±11.8	92.5 ± 10.8	92.3±11.6	89.6 ± 10.9	87.5±9.7	97.7 ± 10.9	93.1±10.9	99.3 ± 13.7	95.3±13.8
Hip circumference (cm)	103.5± 6.7	102.3±6.8	103.5 ± 5.8	102.9±6.1	100.5 ± 5.8	99.7±6.1	104.0 ± 6.0	102.6±6.1	105.6 ± 8.1	104.0±8.0
Waist-Hip ratio	0.92 ± 0.08	0.90±0.08	0.89 ± 0.07	0.89±0.07	0.89 ± 0.09	0.89±0.09	0.94 ± 0.07	0.90±0.07	0.94 ± 0.09	0.91±0.09
Fat body mass (kg)	29.9 ± 8.5	26.0±6.0	26.9 ± 6.1	27.0±7.2	26.7 ± 6.5	23.0±4.5	31.5 ± 8.5	27.1±5	33.3 ± 10.4	27.0±6.4
Fat body mass (%)	39.3 ± 8.8	34.9±6.6	37.3 ± 8.4	37.2±7.0	37.7 ± 9.0	33.3±7.2	40.4 ± 9.0	35.4±6.6	41.3 ± 8.8	34.1±5.5
Fat body mass index (kg/m²)	10.6 ± 3.2	9.3±2.2	9.9 ± 2.6	9.8±2.4	9.6 ± 2.8	8.3±2.1	10.8 ± 2.8	9.3±1.6	12.0 ± 3.8	9.8±2.5
Visceral adipose tissue (g)	796.8 ± 401.4	645.9±326.7	701.0 ± 273.0	709.5±378.4	660.5 ± 271.2	503.8±174.5	835.4 ± 465.6	660.4±351.5	949.8 ± 477.1	706.5±350
Lean body mass (kg)	44.2 ± 11.7	47.1±11.6	44.0 ± 12.0	43.5±10.3	42.9 ± 10.7	44.8±9.6	45.2 ± 14.1	49.0±14.2	44.6 ± 10.8	50.0±11.4
Lean body mass index (kg/m²)	15.4 ± 2.9	16.5±2.9	15.7 ± 3.2	15.6±2.8	15.0 ± 2.5	15.8±2.1	15.0 ± 3.0	16.3±3.1	15.8 ± 2.9	17.8±3.0
Bone mineral content (g)	2280±455	2288±449	2234±450	2200±437	2183± 371	2204±378	2375± 544	2371±535	2316 ± 458	2353±446
Bone mineral density (g/cm²)	1.10 ± 0.10	1.11±0.10	1.11 ± 0.13	1.10±0.11	1.08 ± 0.08	1.09±0.08	1.11 ± 0.10	1.11±0.11	1.12 ± 0.10	1.13±0.11

Physical activity and sedentary time parameters										
Valid days (days)	6.8 ± 0.6	6.9±0.5	7.0 ± 0.0	6.7±0.9	6.5 ± 0.9	6.8±0.6	6.8 ± 0.6	7.0±0.0	6.9 ± 0.6	6.9±0.3
Waking time (hours/day)	17.0 ± 0.7	16.7±0.9	16.9 ± 0.8	16.3±1.3	16.9 ± 0.6	16.7±0.7	17.2 ± 0.8	16.9±0.9	17.2 ± 0.7	16.9±0.7
Sleeping time (hours/day)	5.9 ± 0.8	6.2±0.8	6.0 ± 0.8	6.5±1.0	6.0 ± 0.8	6.2±0.7	6.0 ± 0.8	6.1±0.9	5.6 ± 0.8	6.1±0.7
Sedentary time (min/day)	753.1 ± 87.9	731.2±97.0	763.5 ± 59.4	750.2±80.9	746.8 ± 107.3	728.2±120.4	773.1 ± 87.8	762.9±95.6	733.6 ± 89.1	692.2±77.3
LPA (min/day)	173.4 ± 46.6	167.1±43.4	162.6 ± 35.2	149.3±29.1	171.8 ± 60.6	164.3±49.3	166.5 ± 46.7	157.9±46.6	189.0 ± 38.7	191.2±36.1
MPA (min/day)	93.9 ± 36.4	103.1±52.2	85.5 ± 25.6	77.6±23.7	91.9 ± 45.7	110.5±80.7	88.2 ± 36.9	88.7±38.4	107.0 ± 32.5	128.0±31.2
VPA (min/day)	1.8 ± 2.4	1.9±2.3	1.6 ± 1.9	1.0±0.9	1.1 ± 1.0	1.9±3.0	2.2 ± 3.8	2.5±3.0	2.1 ± 2.0	2.0±1.3
MVPA (min/day)	95.6 ± 36.9	105.0±53.5	87.1 ± 25.8	78.6±23.9	93.0 ± 46.3	112.5±83.5	90.4 ± 37.4	91.2±38.8	109.2 ± 33.2	129.9±31.9
TPA	269.0 ± 77.5	272.1±88.3	249.7 ± 55.4	227.9±42.2	264.8 ± 101.4	276.8±122.6	256.9 ± 78.1	249.1±77.9	298.2 ± 62.9	321.1±62.1
Dietary intake parameters										
Energy (kcal/day)	2063 ± 461	2098±492	2023 ± 497	2065±580	2008 ± 385	2063±605	2140 ± 526	2176±360	2069 ± 464	2080±453
Fat (g/day)	87.3 ± 23.5	91.7±27.4	86.4 ± 18.7	91.5±28.5	85.3 ± 27.0	91.9±34.9	87.2 ± 27.3	94.5±22.9	89.8 ± 21.4	89.2±25.3
Protein (g/day)	82.9 ± 25.1	50.8±34.6	72.2 ± 17.7	53.6±36.9	84.7 ± 32.0	52.1±34.6	87.3 ± 25.4	52.9±39.2	85.3 ± 22.5	45.5±30.7
Carbohydrate (g/day)	214.6 ± 59.8	45.7±116.0	217.7 ± 87.3	56.8±144.0	206.0 ± 40.3	41.9±124.8	228.0 ± 53.7	43.4±95.7	207.2 ± 57.0	42.8±112.2

Data are shown as means ± standard deviation. Abbreviations: PAR; Physical Activity Recommendations for adults proposed by the World Health Organization group, HIIT; High Intensity Interval Training group, HIIT+EMS; HIIT plus Whole-Body Electromyostimulation group, LPA; Light Physical Activity, MPA; Moderate Physical Activity, VPA; Vigorous Physical Activity, MVPA; Moderate-Vigorous Physical Activity, TPA; Total Physical Activity.

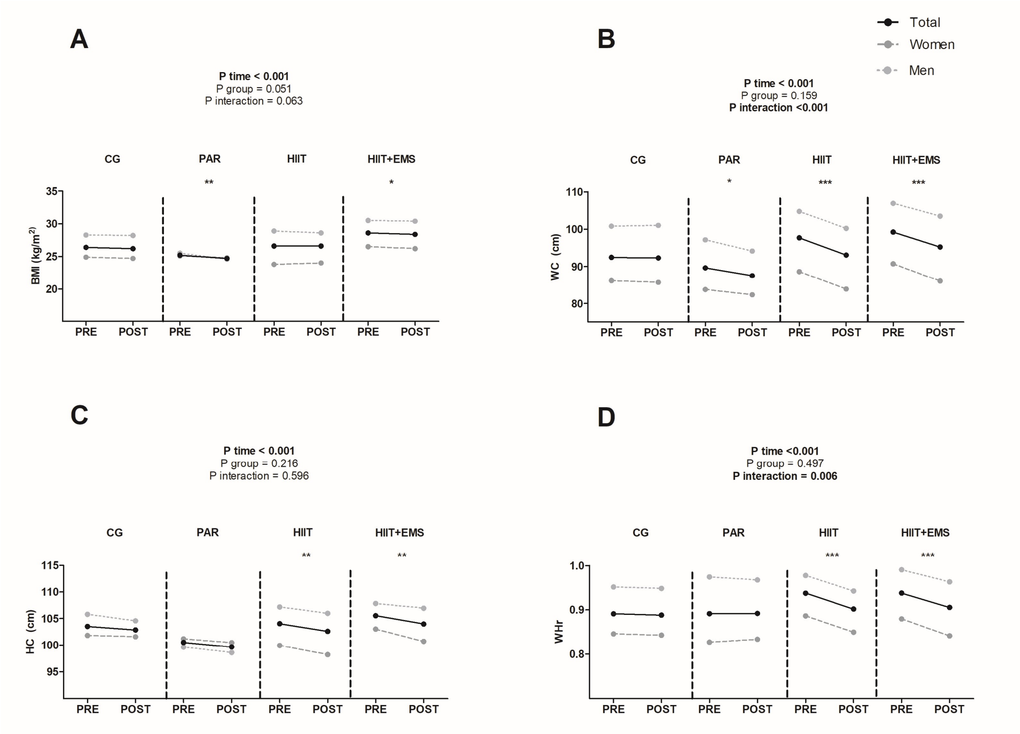

Figure 2. Changes in body mass index (A), waist circumference (B), hip circumference (C) and waist-hip ratio (D) before and after the intervention study. P value (time, group, and interaction [time*group]) of repeated measures ANOVA. * P <0.05; ** P < 0.01; *** P < 0.001 obtained by Student's paired t-test. Abbreviations: PAR; Physical Activity Recommendations for adults proposed by the World Health Organization group, HIIT; High Intensity Interval Training group, HIIT+EMS; HITT plus Whole-Body Electromyostimulation group, BMI; Body Mass Index, WC; Waist Circumference, HC; Hip Circumference, Whir; Waist-Hip Ratio.

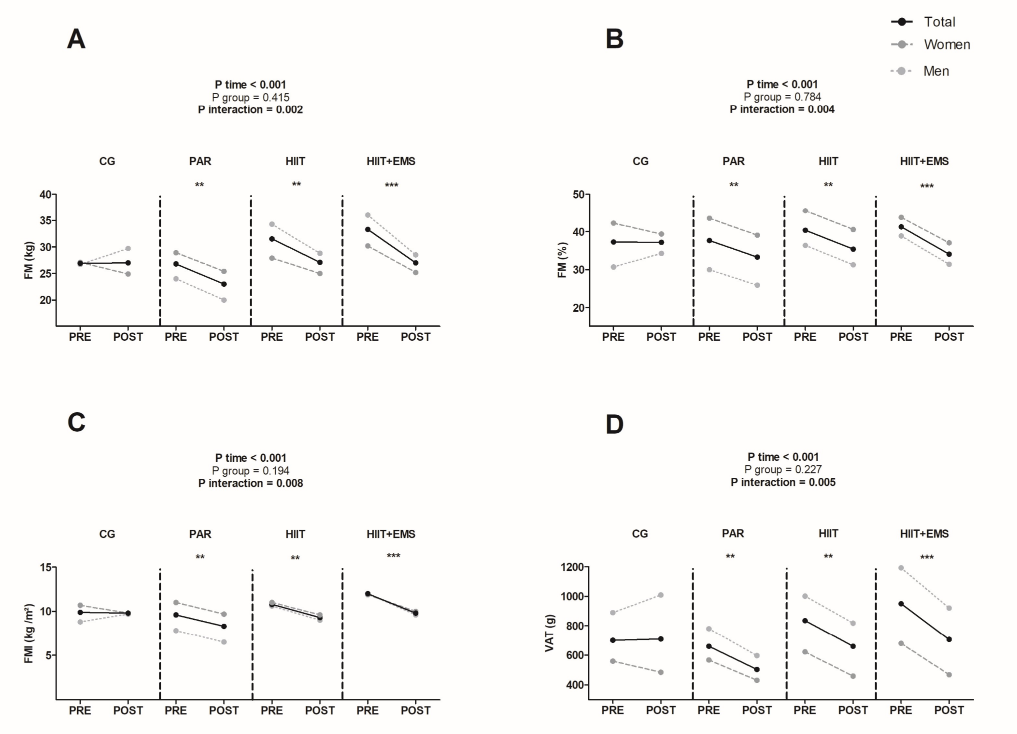

Figure 3. Changes in fat body mass (A), fat body mass percentage (B), fat body mass index (C) and visceral adipose tissue (D) values before and after the intervention study. P value (time, group, and interaction [time*group]) of repeated measures ANOVA. * P <0.05; ** P < 0.01; *** P < 0.001 obtained by Student's paired t-test. Abbreviations: PAR; Physical Activity Recommendations for adults proposed by the World Health Organization group, HIIT; High Intensity Interval Training group, HIIT+EMS; HIIT plus Whole-Body Electromyostimulation group, FM; Fat Body Mass; FMI; Fat Body Mass Index, VAT; Visceral Adipose Tissue.

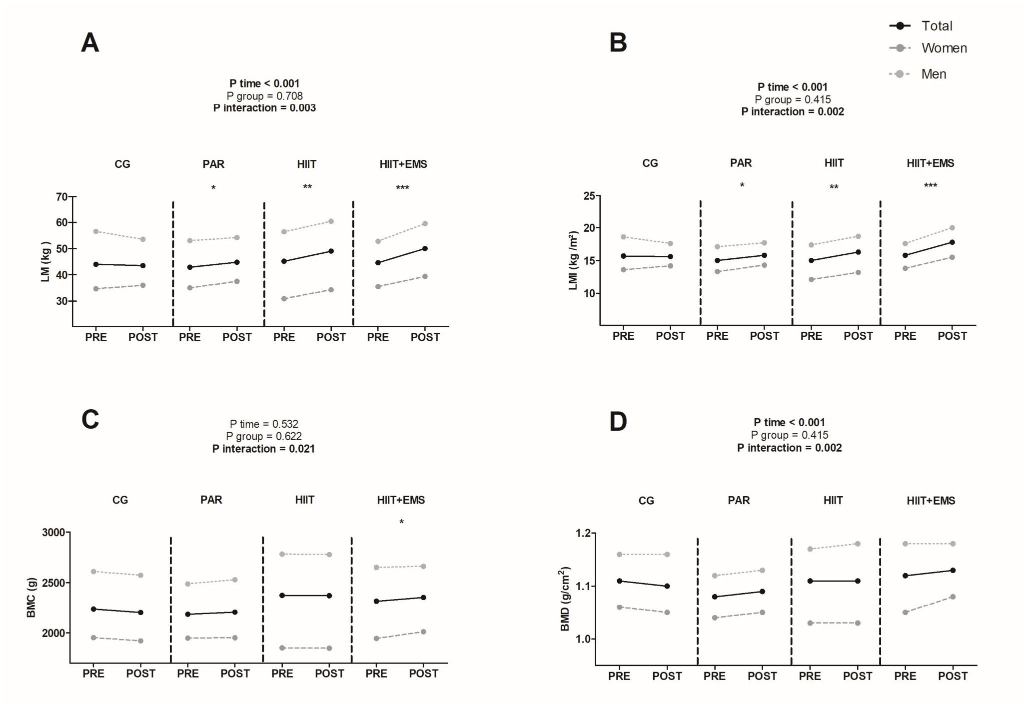

Figure 4. Changes in lean body mass (A), lean body mass index (B), bone mineral content (C) and bone mineral density (D) values before and after the intervention study. P value (time, group, and interaction [time*group]) of repeated measures ANOVA. * P <0.05; ** P < 0.01; *** P < 0.001 obtained by Student's paired t-test. Abbreviations: PAR; Physical Activity Recommendations for adults proposed by the World Health Organization group, HIIT; High Intensity Interval Training group, HIIT+EMS; HIIT plus Whole-Body Electromyostimulation group, LM; Lean Body Mass, LMI; Lean Body Mass Index, BMC; Bone Mineral Content, BMD; Bone Mineral Density.

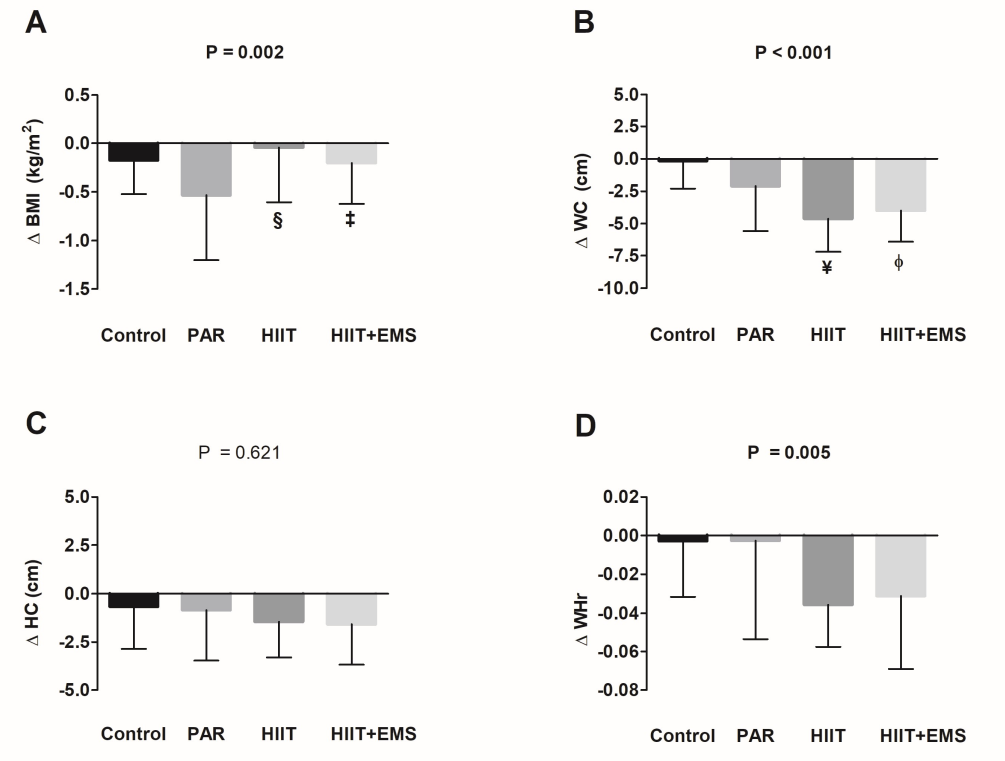

Figure 5. Changes in body mass index (A), waist circumference (B), hip circumference (C) and waist-hip ratio (D) after the intervention study among the four groups. Data are shown as means ± standard deviation. ¥ P<0.05 Control vs. HIIT; ϕ P<0.05 Control vs. HIIT+EMS; § P<0.05 PAR vs. HIIT; ‡ P<0.05 PAR vs. HIIT+EMS, ANCOVA adjusting by baseline values, with post hoc Bonferroni-corrected t-test. Abbreviations: PAR; Physical Activity Recommendations for adults proposed by the World Health Organization group, HIIT; High Intensity Interval Training group, HIIT+EMS; HIIT plus Whole-Body Electromyostimulation group, BMI; Body Mass Index, WC; Waist Circumference, HC; Hip Circumference; Whir; Waist-Hip Ratio.

Table 2. Changes in body composition outcomes adjusted by baseline values (Model 1) adjusted by baseline values and sex (Model 2), by baseline values and age (Model 3), by baseline values and total physical activity changes (Model 4), and by baseline values and total energy intake changes (Model 5).

	ANCOVA P value				
	Model 1	Model 2	Model 3	Model 4	Model 5
BMI (kg/m²)	**0.002**	**0.005**	**0.002**	**0.002**	**0.003**
WC (cm)	**<0.001**	**<0.001**	**<0.001**	**0.001**	**<0.001**
HC (cm)	0.621	0.679	0.758	0.630	0.879
WHr	**0.005**	**0.003**	**0.012**	**0.013**	**0.007**
FM (kg)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
FM (%)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
FMI (kg/m²)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
VAT (g)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
LM (kg)	**0.001**	**<0.001**	**<0.001**	**0.047**	**0.010**
LMI (kg/m²)	**<0.001**	**<0.001**	**<0.001**	**0.012**	**0.003**
BMC (g)	**0.020**	**0.030**	**0.041**	0.094	**0.049**
BMD (g/cm²)	0.179	0.250	0.273	0.200	0.290

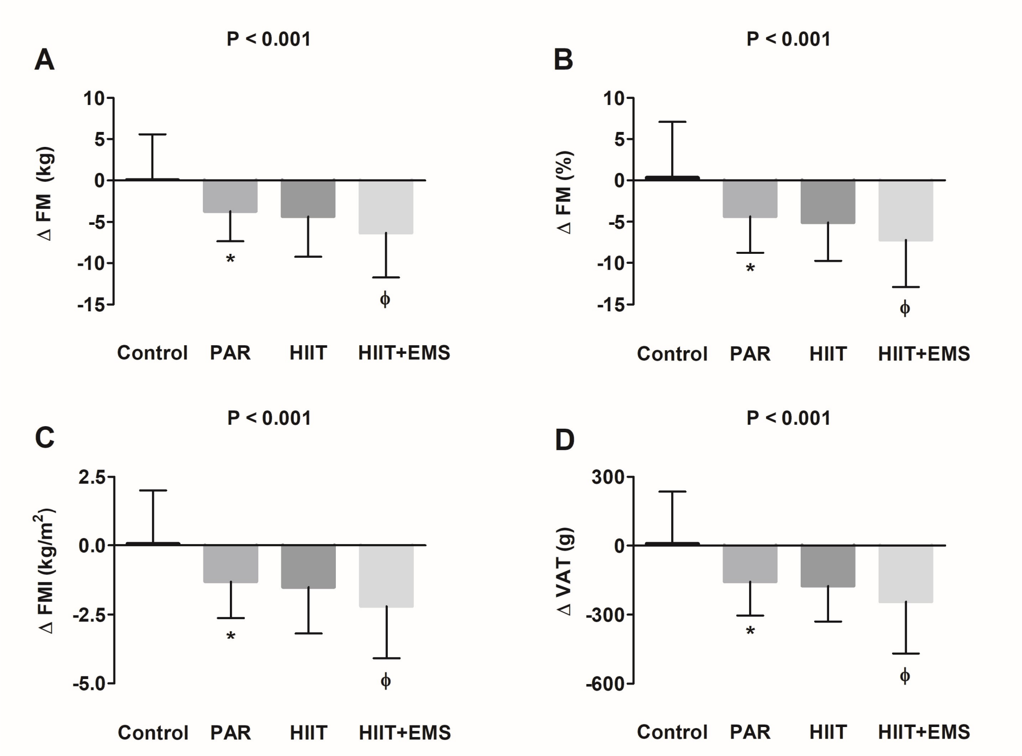

Figure 6. Changes in fat body mass (A) fat body mass percentage (B), fat body mass index (C) and visceral adipose tissue (D) after the intervention study among the four groups. Data are shown as means ± standard deviation, unless panel A, shown as changes from baseline (%) ± standard deviation. * P<0.05, Control vs. PAR; ϕ P<0.05 Control vs. HIIT+EMS, ANCOVA adjusting by baseline values, with post hoc Bonferroni-corrected t-test. Abbreviations: PAR; Physical Activity Recommendations for adults proposed by the World Health Organization group, HIIT; High Intensity Interval Training group, HIIT+EMS; HIIT plus Whole-Body Electromyostimulation group, FM; Fat Body Mass, FMI; Fat Body Mass Index, VAT; Visceral Adipose Tissue.

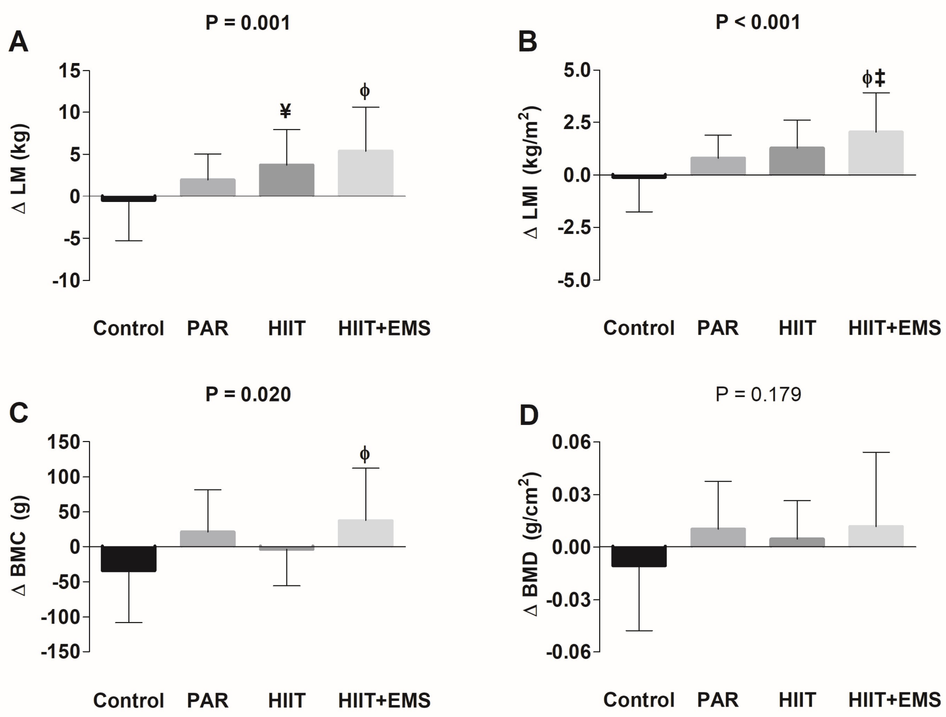

Figure 7. Changes in lean body mass (A), lean body mass index (B), bone mineral content (C), bone mineral density (D) after the intervention study among the four groups. Data are shown as means ± standard deviation. ¥ P<0.05 Control vs. HIIT; ϕ P<0.05 Control vs. HIIT+EMS; ‡ P<0.05 PAR vs. HIIT+EMS, ANCOVA adjusting by baseline values, with post hoc Bonferroni-corrected t-test. Abbreviations: PAR; Physical Activity Recommendations for adults proposed by the World Health Organization group, HIIT; High Intensity Interval Training group, HIIT+EMS; HIIT plus Whole-Body Electromyostimulation group, LM; Lean Body Mass, LMI; Lean Body Mass Index, BMC; Bone Mineral Content, BMD; Bone Mineral Density.

It has been previously reported that concurrent training induces a decrease of FM and an increase of LM in sedentary men after 3 sessions/week (55-70% of VO_2max intensity for endurance training, and 65-85 % of 1RM intensity for resistance training) during a 24-weeks intervention (-4% in FM and +2% in LM) [2], and in sedentary women after 3 sessions/week (that included endurance training at moderate intensity, and 2 rounds of 9 resistance exercises with 1 set of 8–12 repetition maximum) during 10 weeks (-5% in FM and +8% in LM) [3]. These results agree with our findings, since a significant decrease of FM and a significant increase of LM (-4% in FM and +4% in LM) were obtained in PAR.

A recent meta-analysis (which included a total of 39 studies) concluded that HIIT significantly reduces FM (-6%), especially VAT (-8%) in sedentary individuals with a BMI ranged from 18.5 to 35 kg/m^2. These results are quite similar than those obtained by Wewege et al. [7] in a systematic review and meta-analysis that studied the effects of moderate intensity continuous exercise programs vs. HIIT programs on FM in sedentary individuals, concluding that both exercise training programs appear to be similarly effective on FM reduction (-7% in both cases) after a ~10-weeks training program. However, no changes in LM were observed neither after moderate intensity continuous exercise programs nor after HIIT programs [7]. Moreover, Nybo et al. [8] also compared a 12-weeks moderate intensity continuous exercise program vs. 12-weeks HIIT program in untrained men, showing no significant changes in LM and in BMC in both cases. However, they included an additional resistance training exercise program which performed 3-4 sets of strength exercises with an intensity of 12 to 16 repetition maximum (2 sessions/week): this group showed a significant increase of both LM (+3%) and BMC (+2%) [7]. These results agree with those obtained by Kukuljan et al. [4] and Sañudo et al. [5] that showed a significant increase of BMD after a resistance training program in sedentary men and women (+1-2% in both cases). Despite the significant decrease of FM and increase of LM found in the HIIT group (-5% and +5%, respectively), partly consistent with previous findings [2,4,7,8,40], no significant changes were noted in BMC. The lack of improvements in BMC can be attributed to the short duration of our intervention program, since longer exercise training program are usually required to improve BMC [4,5].

A number of studies have previously examined the role of WB-EMS on body composition parameters, showing that this training modality induced a decrease of FM and an increase of LM in individuals with different ages and biological characteristics [10,11,14,17,19,21,23,26,28,29]. Curiously, the exercise training methodology proposed in all of these studies was similar: (i) 10–14 dynamic exercises (without any additional weights) structured in 1-2 sets of 8 repetitions, (ii) impulse frequency of 85 Hz, (iii) impulse width of 350 µs, (iv) duty cycle of 50%.

Despite the different exercise training program durations (ranged from 14 to 55 weeks) and the fact that these WB-EMS programs were performed in individuals with different ages and biological characteristics, they showed a decrease of FM (ranged from -8.5 to -0.5% of FM), an increase of LM (ranged from +0.5 to +2.5% of LM), and an slight increase of BMD (+0.5%) [10,11,14,17,19,21,23,26,28,29]. These results concur with our findings that revealed a significant decrease of -7% in FM, a significant increase of +7% in LM and a significant increase of +1.5% in BMC. The reasons why we obtained similar improvements in body composition after the application of short-term WB-EMS programs than the above-mentioned studies (12 weeks vs. 14 to 55 weeks) could be that: (i) Our study participants were sedentary middle-aged adults, whereas the majority of the previous studies included sedentary older adults with obesity, sarcopenic obesity and/or osteopenia. (ii) We designed a periodized and functional exercise program based on HIIT adding WB-EMS, while the previous studies applied a low-intensity resistance program with WB-EMS (6-9 vs. 1-7 of the ratings of perceived exertion scale [34]). (iii) We applied different electrical parameters compared with those selected by the above-mentioned studies, taking into account the biological characteristics of our study participant's and based on the scientific evidence [41], aiming to maximize the positive effects induced by this training modality.

The additional body composition improvements obtained by the HIIT+EMS group (specially in LM) could be consequence of a greater number of muscle contractions promoting a higher muscle mechanical tension, muscle damage, and muscle metabolic stress which are the main mechanism of muscle hypertrophy [42].

Limitations

This study had a number of limitations. Due to the limited sample size, our study might have been underpowered to detect statistical differences in specific body composition parameters between the different training modalities, although we found a decrease of FM, FM percentage, FMI and VAT, and an increase of LM and LMI in the PAR group as well as in the HIIT group and in the HIIT+EMS group compared with the control group. Our study only included sedentary middle-aged adults, and hence we cannot extend these findings to older, younger, and/or physically active individuals.

CONCLUSION

In conclusion, our results show that the PAR group, the HIIT group and the HIIT+EMS group induced a decrease of FM related parameters compared to the control group, while only the HIIT group and the HIIT+EMS group showed an increase of LM related parameters compared to the control group in sedentary middle-aged adults. Moreover, a significantly increase of BMC were observed in the HIIT+EMS group compared to the control group in sedentary middle-aged adults.

Perspectives

Our findings suggest that a HIIT program adding or not WB-EMS, as well as a concurrent training programs based on physical activity recommendation from the World Health Organization can be used as a strategy to improve body composition parameters related to high-incidence pathologies such us obesity, sarcopenia and osteoporosis, obtaining slightly better results with the application of a HIIT+EMS program (specially in term of LM and BMC). Moreover, when designing a WB-EMS program, we encourage to consider the biological characteristics of the study participant's (age, sex, or training status among others), since its optimization in terms of electrical parameters (i.e. impulse frequency, impulse intensity, impulse breadth and duty cycle) may increase its effects on body composition and health-related parameters.

REFERENCES

1 Hita-Contreras F, Martínez-Amat A, Cruz-Díaz D, Pérez-López FR. Osteosarcopenic obesity and fall prevention strategies. *Maturitas* 2015; **80**: 126–132.

2 Michell V, Samaria C, Júnior Rudy N, Danyela V, Dantas E. Effects of a concurrent physical exercise program on aerobic power and body composition in adults. *J Sports Med Phys Fitness* 2014; **54**: 441–6.

3 Ferreira FC, de Medeiros AI, Nicioli C, Nunes JED, Shiguemoto GE, Prestes J *et al.* Circuit resistance training in sedentary women: body composition and serum cytokine levels. *Appl Physiol Nutr Metab* 2010; **35**: 163–171.

4 Kukuljan S, Nowson CA, Sanders KM, Nicholson GC, Seibel MJ, Salmon J *et al.* Independent and combined effects of calcium-vitamin D3 and exercise on bone structure and strength in older men: An 18-month factorial design randomized controlled trial. *J Clin Endocrinol Metab* 2011; **96**: 955–963.

5 Sañudo B, de Hoyo M, del Pozo-Cruz J, Carrasco L, del Pozo-Cruz B, Tejero S *et al.* A systematic review of the exercise effect on bone health. *Menopause* 2017; **24**: 1.

6 Trost SG, Owen N, Bauman AE, Sallis JF, Brown W. Correlates of adults' participation in physical activity: review and update. *Med Sci Sports Exerc* 2002; **34**: 1996–2001.

7 Wewege M, van den Berg R, Ward RE, Keech A. The effects of high-intensity interval training vs. moderate-intensity continuous training on body composition in overweight and obese adults: a systematic review and meta-analysis. *Obes Rev* 2017; **18**: 635–646.

8 Nybo L, Sundstrup E, Jakobsen MD, Mohr M, Hornstrup T, Simonsen L *et al.* High-intensity training versus traditional exercise interventions for promoting health. *Med Sci Sports Exerc* 2010; **42**: 1951–1958.

9 Kemmler W, Weissenfels A, Willert S, Shojaa M, von Stengel S, Filipovic A *et al.* Efficacy and safety of low frequency Whole-Body Electromyostimulation (WB-EMS) to improve health-related outcomes in non-athletic adults. A systematic review. *Front Physiol* 2018; **9**.

10 Kemmler W, Kohl M, Freiberger E, Sieber C, Von Stengel S. Effect of whole-body electromyostimulation and / or protein supplementation on obesity and cardiometabolic risk in older men with sarcopenic obesity: The randomized controlled FranSO trial. *BMC Geriatr* 2018; **18**: 1–12.

11 Kemmler W, Grimm A, Bebenek M, Kohl M, von Stengel S. Effects of Combined Whole-Body Electromyostimulation and Protein Supplementation on Local and Overall Muscle/Fat Distribution in Older Men with Sarcopenic Obesity: The Randomized Controlled Franconia Sarcopenic Obesity (FranSO) Study. *Calcif*

Tissue Int 2018; **103**: 1–12.

12 Filipovic A, Grau M, Kleinöder H, Zimmer P, Hollmann W, Bloch W. Effects of a Whole-Body Electrostimulation Program on Strength, Sprinting, Jumping, and Kicking Capacity in Elite Soccer Players. *J Sports Sci Med* 2016; **15**: 639–648.

13 Boccia G, Fornasiero A, Savoldelli A, Bortolan L, Rainoldi A, Schena F *et al.* Oxygen consumption and muscle fatigue induced by whole-body electromyostimulation compared to equal-duration body weight circuit training. *Sport Sci Health* 2016.

14 Von Stengel S, Bebenek M, Engelke K, Kemmler W. Whole-Body Electromyostimulation to Fight Osteopenia in Elderly Females: The Randomized Controlled Training and Electrostimulation Trial (TEST-III). *J Osteoporos* 2015; **11**: 1–7.

15 Kemmler, Teschler M, Bebenek M, von Stengel S. [(Very) high Creatinkinase concentration after exertional whole-body electromyostimulation application: health risks and longitudinal adaptations]. *Wien Med Wochenschr* 2015; **165**: 427–35.

16 Filipovic A, Kleinöder H, Plück D, Hollmann W, Bloch W, Grau M. Influence of Whole-Body Electrostimulation on Human Red Blood Cell Deformability. *J strength Cond Res* 2015; **29**: 2570–8.

17 Kemmler W, Bebenek M, Engelke K, von Stengel S. Impact of whole-body electromyostimulation on body composition in elderly women at risk for sarcopenia: the Training and ElectroStimulation Trial (TEST-III). *Age (Dordr)* 2014; **36**: 395–406.

18 Van Buuren F, Mellwig KP, Prinz C, Körber B, Fründ A, Fritzsche D *et al.* Electrical myostimulation improves left ventricular function and peak oxygen consumption in patients with chronic heart failure: Results from the exEMS study comparing different stimulation strategies. *Clin Res Cardiol* 2013; **102**: 523–534.

19 Kemmler, von Stengel S. Whole-body electromyostimulation as a means to impact muscle mass and abdominal body fat in lean, sedentary, older female adults: subanalysis of the TEST-III trial. *Clin Interv Aging* 2013; **8**: 1353–64.

20 Kemmler, Von Stengel S, Schwarz J, Mayhew JL. Effect of whole-body electromyostimulation on energy expenditure during exercise. *J Strength Cond Res* 2012; **26**: 240–5.

21 Kemmler, Schliffka R, Mayhew JL, von Stengel S. Effects of whole-body electromyostimulation on resting metabolic rate, body composition, and maximum strength in postmenopausal women: the Training and ElectroStimulation Trial. *J strength Cond Res* 2010; **24**: 1880–7.

22 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.

23 Amaro-Gahete F, De-la-O A, Robles-González L, Castillo M, Gutierrez A. Impact of two whole-body electromyostimulation training modalities on body composition in recreational runners during endurance training cessation. *Rev Int Cienc Deporte* 2018; **53**: 205–218.

24 De La Camara MA, Pardos AI, Veiga ÓL. Effectiveness evaluation of whole-body electromyostimulation as a post-exercise recovery method. *J Sports Med Phys Fitness* 2018.

25 Teschler M, Wassermann A, Weissenfels A, Fröhlich M, Kohl M, Bebenek M *et al.* Short time effect of a single session of intense whole-body electromyostimulation on energy expenditure. A contribution to fat reduction? *Appl Physiol Nutr Metab* 2018; **530**: 1–3.

26 Kemmler W, Weissenfels A, Teschler M, Willert S, Bebenek M, Shojaa M *et al.* Whole-body electromyostimulation and protein supplementation favorably affect sarcopenic obesity in community-dwelling older men at risk: The randomized controlled FranSO study. *Clin Interv Aging* 2017; **12**: 1503–1513.

27 Wittmann K, Sieber C, Von Stengel S, Kohl M, Freiberger E, Jakob F *et al.* Impact of whole body electromyostimulation on cardiometabolic risk factors in older women with sarcopenic obesity: The randomized controlled FORMOsA-sarcopenic obesity study. *Clin Interv Aging* 2016; **11**: 1697–1706.

28 Kemmler, Teschler M, Weißenfels A, Bebenek M, Fröhlich M, Kohl M *et al.* Effects of Whole-Body Electromyostimulation versus High-Intensity Resistance Exercise on Body Composition and Strength: A Randomized Controlled Study. *Evidence-based Complement Altern Med* 2016; **3**: 44–55.

29 Kemmler, Teschler M, Weissenfels A, Bebenek M, von Stengel S, Kohl M *et al.* Whole-body electromyostimulation to fight sarcopenic obesity in community-dwelling older women at risk. Results of the randomized controlled FORMOsA-sarcopenic obesity study. *Osteoporos Int* 2016; **27**: 3261–3270.

30 Storlien L, Oakes ND, Kelley DE. Metabolic flexibility. *Proc Nutr Soc* 2004; **63**: 363–8.

31 Schulz KF, Altman DG, Moher D, Jüni P, Altman D, Egger M *et al.* CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMC Med* 2010; **8**: 18.

32 WHO. Global recommendations on physical activity for health, World Health Organ publications. Geneva, Switzerland, 2015.

33 Buchheit M, Laursen PB. High-intensity interval training, solutions to the programming puzzle: Part I:

Cardiopulmonary emphasis. *Sport Med* 2013; **43**: 313–338.

34 Borg GA. Psychophysical bases of perceived exertion. *Med Sci Sports Exerc* 1982; **14**: 377–81.

35 Migueles JH, Cadenas-Sanchez C, Ekelund U, Delisle Nyström C, Mora-Gonzalez J, Löf M *et al.* Accelerometer Data Collection and Processing Criteria to Assess Physical Activity and Other Outcomes: A Systematic Review and Practical Considerations. *Sport Med* 2017; **47**: 1821–1845.

36 van Hees VT, Fang Z, Langford J, Assah F, Mohammad A, da Silva ICM *et al.* Autocalibration of accelerometer data for free-living physical activity assessment using local gravity and temperature: an evaluation on four continents. *J Appl Physiol* 2014; **117**: 738–44.

37 Van Hees VT, Sabia S, Anderson KN, Denton SJ, Oliver J, Catt M *et al.* A novel, open access method to assess sleep duration using a wrist-worn accelerometer. *PLoS One* 2015; **10**: 1–13.

38 Hildebrand M, Hansen BH, van Hees VT, Ekelund U. Evaluation of raw acceleration sedentary thresholds in children and adults. *Scand J Med Sci Sport* 2017; **27**: 1814–1823.

39 Baugh E, Hedrick VE, Marinik EL, Kyle D. Dietary Intake Modification in Response to Participation in a Resistance Training Program for Sedentary Older Adults with Prediabetes: Findings from the Resist Diabetes Study. *Eat Behav* 2015; **15**: 379–382.

40 Maillard F, Pereira B, Boisseau N. Effect of High-Intensity Interval Training on Total, Abdominal and Visceral Fat Mass: A Meta-Analysis. *Sport Med* 2018; **48**: 269–288.

41 Filipovic A, Kleinöder H, Dörmann U, Mester J. Electromyostimulation--a systematic review of the influence of training regimens and stimulation parameters on effectiveness in electromyostimulation training of selected strength parameters. *J strength Cond Res* 2011; **25**: 3218–38.

42 Schoenfeld BJ. The mechanisms of muscle hypertrophy and their application to resistance training. *J strength Cond Res* 2010; **24**: 2857–72.

Chapter 11:
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 randomised controlled trial
(Study 15)

ABSTRACT

This study aimed to investigate the influence of different exercise training modalities ([i] PAR group, [ii] a HIIT group, and [iii] a HIIT+EMS group) on physical fitness in sedentary middle-aged adults.

A total of 89 (52.7% women) middle-aged sedentary adults (53.7±5.1 years old) were enrolled in the FIT-AGEING study. Cardiorespiratory fitness was determined by a maximum treadmill test using indirect calorimetry. Lower, upper, and core body muscular strength were assessed by an isokinetic strength test, by the handgrip strength test, and by several core strength endurance tests, respectively.

All the exercise types induced similar increases on cardiorespiratory fitness (Δ VO_2max≥11%, Δ maximal heart rate≥8%, and Δ total test duration ≥14%; all P≤0.034), as well as on muscular strength (Δ extension and flexion peak torque≥10%, Δ total hand grip ≥3%, Δ core strength endurance tests≥20%; all P≤0.050) compared with a control group.

In conclusion, our results suggest that a 12-week structured exercise intervention improves physical fitness regardless of the training programme in sedentary middle-aged adults. Despite slightly greater improvements in some physical fitness variables, the changes observed in the HIIT+EMS group were not superior to the other exercise programmes.

BACKGROUND

Cardiorespiratory fitness and muscular strength have been positioned as two independent powerful health markers [1]. Epidemiological studies have indicated an inverse association of VO_2max with coronary heart disease, cardiovascular disease events, different types of cancer, and all-cause mortality in both men and women of different ages, which is unaffected by different factors, such as alcohol or tobacco consumption [2,3]. Furthermore, a recent review article proposed that muscular strength is negatively associated with all-cause mortality even after controlling for physical activity levels and VO_2max [4–6].

Several studies have shown that physical exercise is an effective strategy to fight against the high prevalence of chronic diseases [7], improving physical fitness, and, consequently, increasing quality of life [5,8–13]. It is well-known that the application of different training modalities produce important, but not similar health-related physiological adaptations [14,15]. The World Health Organization recommended performing concurrent training combining endurance (>150 min/week) and resistance training (>2 sessions/week) [16]. Unfortunately, the lack of free time is the principal barrier to do exercise in developed countries [17]. In this context, alternative and less time-consuming training methodologies that allow us to maximize the potential benefits induced by exercise have recently emerged.

HIIT has been positioned as an efficient alternative [18] to induce improvements on VO_2max [19–21] and muscular strength [22,23] simultaneously [23], offering potentially better results in older and less fit individuals [23]. Although HIIT has been considered the most popular time-efficient exercise methodology, new training tendencies are emerging. Several studies have recently investigated the effects of WB-EMS on health-related parameters [24–33]. WB-EMS is a novel training technology that simultaneously innervates up to 12 main muscle groups with a specific electrical intensity. Previous studies have investigated its effects on physical fitness in trained and untrained individuals showing that this training methodology induced a general increase in maximum dynamic and isometric leg-press strength, vertical jump performance, and maximum hand grip strength [24–33]. Furthermore, an increment in VO_2max has recently been reported after a 6-week WB-EMS programme in recreational runners [24,25].

Little is known about whether different exercise training methodologies could induce different effects on health-related parameters. In this sense, Kemmler et al. compared the influence of a HIIT programme versus a WB-EMS programme. The authors concluded that both training methodologies were equally effective to improve the cardio-metabolic risk profile in sedentary middle-aged men [32]. However, there are no studies that compare the effects of different exercise training methodologies on physical fitness in sedentary middle-aged adults. Thus, the purpose of this study was to compare the influence of traditional concurrent training vs. HIIT adding or not WB-EMS on physical fitness in sedentary middle-aged adults.

MATERIAL & METHODS

Participants

A total of 89 participants (52.7% women) were assessed for eligibility following recruitment via social networks, local media, and posters. Prior to the enrolment, all potential individuals completed a medical examination to identify any pathological condition and current medication that could affect the ability to complete the required exercise training and testing. The inclusion criteria were as follows: (i) adults aged between 45 and 65 years old, (ii) not to be physically active (<20 minutes of moderate-intensity physical activity on 3 days/week over the previous three months), (iii) to have a stable weight during the previous 6 months (weight changes <3 kg), and (iv) not to have a history of cardiovascular disease, diabetes mellitus, cancer, and/or major illness (acute or chronic) including any that can limit the ability to complete the necessary exercises. A total of 15 participants dropped out between the randomisation and the follow-up due to (i) not having time (n=6), (ii) medical reasons (n=2), (iii) job related relocation (n=3), and (iv) other reasons (n=4). A total of 74 participants were included in the final analysis. All participants provided a written informed consent to participate in the current study (http://www.clinicaltrials.gov, ID: NCT03334357) which complied with the requirements of the last revised Declaration of Helsinki and was approved by the Human Research Ethics Committee of the "Junta de Andalucía" [0838-N-2017]. Figure 1 shows the flow of participants throughout the study.

Study design

A 12-week randomised controlled trial with a parallel group design following the CONSORT (Consolidated Standards of Reporting Trials) guidelines [34] was conducted. For practical and feasibility reasons, the study was conducted in 2 waves with 45 participants maximum. Following the baseline testing (September 2016 and September 2017, respectively), the participants were allocated into 4 different groups using a computer-generated simple randomisation software [35]: [i] a PAR group, [ii] a HIIT group, and [iii] a HIIT+EMS group. The randomisation process was blinded to the assessment staff. All participants were instructed to maintain their usual physical activity levels and not to engage in other additional structured exercise outside of the intervention programme.

Exercise training programmes

A detailed description of each exercise training programme can be found elsewhere

[36]. An attendance of at least 90% of sessions was required to be included in the final analysis. All training sessions were performed in groups of 2 to 6 participants and a gradual progression was also scheduled in order to ensure a good adherence to each intervention group.

The participants allocated in the PAR group completed 3 concurrent training sessions per week for 12 weeks with at least 48 hours of recovery between each session. A total of 150 min/week at 60-65% of the HRres was established for the endurance training and ~60 min/week at 40-50% of 1RM for the resistant training. Different ergometers (i.e. treadmill, cycle-ergometer, and elliptical ergometer) were selected to conduct the endurance training, and weight bearing and guided pneumatic machines were selected to conduct the resistance training (i.e. squat, bench press, dead lift, or lateral pull down).

The participants allocated in the HIIT group completed 2 sessions/week for 12 weeks with at least 72 h of recovery between each session. The participants followed 2 different and alternative HIIT protocols [37,38], which included a HIIT with long intervals protocol and a HIIT with short intervals protocol. A volume of 40-65 min/week was established at >95% of VO_2max in HIIT with long intervals, and 6-9 of the ratings of perceived exertion scale [39] in HIIT with short intervals. Treadmill with a personalised slope was the exercise modality applied in HIIT with long intervals, and 8 weight-bearing exercises (i.e. squat, dead lift, high knees up, high heels up, push up, horizontal row, lateral plank, and frontal plank) in circuit form was the exercise methodology applied in HIIT with short intervals.

The participants allocated in the HIIT+EMS group completed a training programme with similar characteristics to those used for the HIIT group adding WB-EMS with a wireless device (Wiemspro®, Malaga, Spain).

The electric pulse was bipolar, symmetrical, and rectangular with a frequency of 15-20 hertz in HIIT with long intervals and 35-75 hertz in HIIT with short intervals, an intensity of 100 milliamps in HIIT with long intervals, and 80 milliamps in HIIT with short intervals, an impulse breadth of 200-400 µsec in both in HIIT with long intervals and in HIIT with short intervals (thighs=400 µsec, glutes=350 µsec, abdominals=300 µsec, low back=250 µsec, mid back=250 µsec, high back=200 µsec, chest=200 µsec, and arms=200 µsec), and a duty cycle (ratio of on-time to the total cycle time: % duty cycle = 100/ [total time/on-time]) of 99% in HIIT with long intervals and 50-63% in HIIT with short intervals, considering previous methodological issues [40].

A dynamic standardised warm-up and an active global stretching cooling-down protocol [36] were, respectively, completed at the beginning and at the end of each training session in all intervention groups [36]. An extra effort was made to promote maximal attendance.

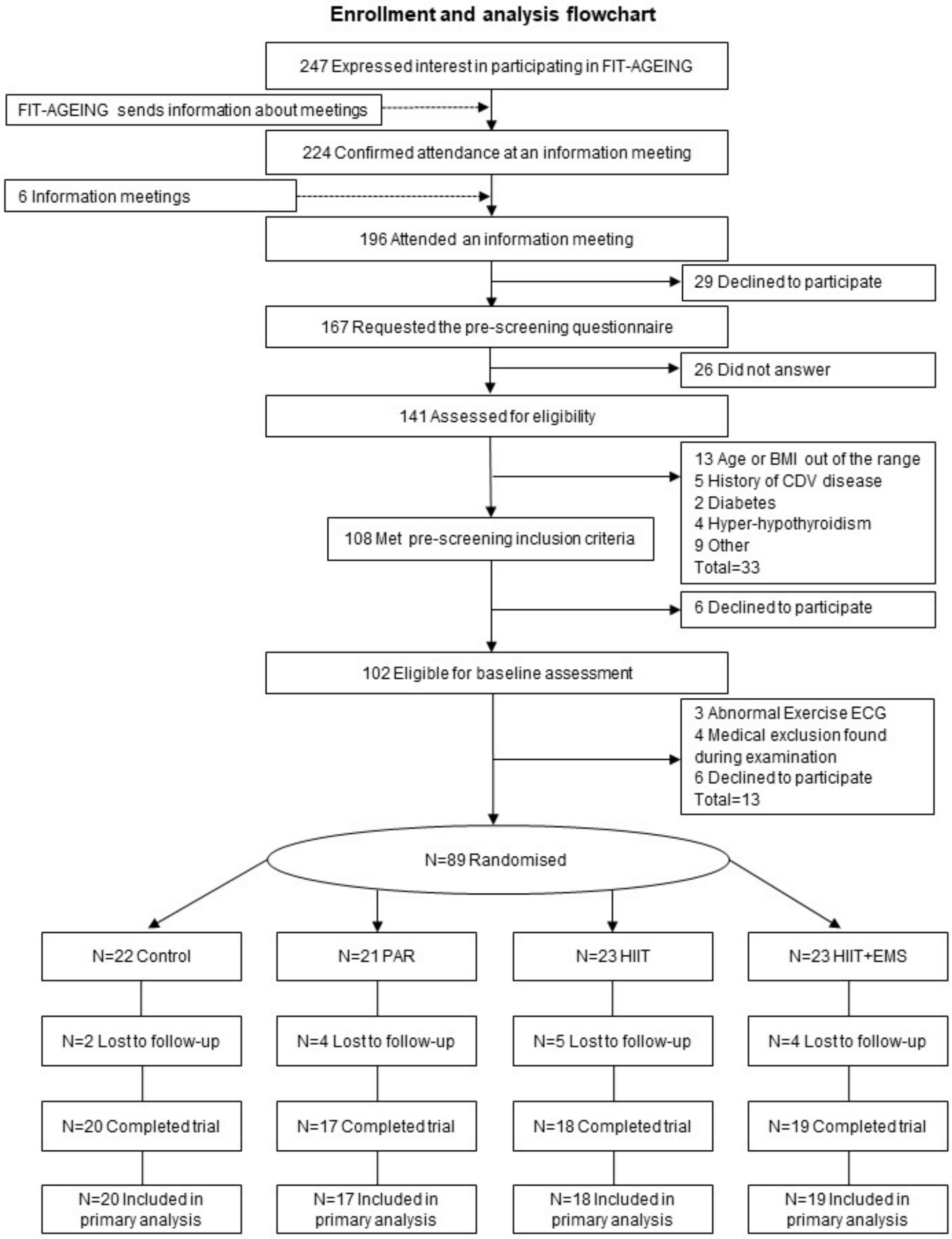

Figure 1: Flow-chart diagram. Abbreviations: BMI; body mass index, CDV; cardiovascular, ECG; electrocardiogram, PAR; Physical Activity Recommendations for adults proposed by the World Health Organization group, HIIT; High Intensity Interval Training group, HIIT+EMS; HIIT plus Whole-Body Electromyostimulation group.

For instance, the sessions were rescheduled when a participant was unable to attend due to work, family, or illness. The participants were constantly motivated throughout each training session and were instructed to reach the specific target intensity. Heart rate was continuously monitored during exercise at 5-second intervals using a pulsometer (Polar RS300, Kempele, Finland).

Anthropometric and body composition assessment

We measured weight and height through a pre-validated scale and stadiometer (model 799, Electronic Column Scale, Hamburg, Germany) with light clothes and barefoot. The BMI was also determined (weight/height^2).

Body composition was measured using a dual-energy X-ray absorptiometry scanner (Discovery Wi, Hologic, Inc., Bedford, MA, USA). A whole-body scan was used to obtain all parameters. FMI and LMI were calculated as FM divided by height^2 (kg/m^2) and LM divided by height^2 (kg/m^2), respectively.

Dietary intake assessment

We performed a total of three 24-hour recalls collected on non-consecutive days (one weekend day included) to determine the dietary intake before and after the intervention programme [41]. Detailed information of the food consumed by the participants was obtained through an interview conducted by qualified nutrition expert. Coloured photographs of different food portions sizes were used to help estimate the quantity of food consumed [42]. We used a specific software (EVALFINUT®, Ibero-American Foundation of Nutrition, Spain) to calculate energy intake and macronutrient content averaging the three 24-hour recalls.

Sedentary time and physical activity assessment

Sedentary time and physical activity levels were assessed with a wrist-worn accelerometer (ActiGraph GT3X+, Pensacola, FL, US) during 7 consecutive days (24 hours/day) before and after the intervention [36]. The ActiLife v.6.13.3 software (ActiGraph, Pensacola, FL, US) and the GGIR package (v. 1.5-12, https://cran.r-project.org/web/packages/GGIR/) in R (v. 3.1.2, https://www.cran.r-project.org/) was used to process these files [43,44]. The participants that did not wear the accelerometers for at least 16 hours/day during 4 days were discarded.

Physical fitness assessment

A maximum treadmill (H/P/Cosmos Pulsar treadmill, H/P/Cosmos Sport & Medical GMBH, Germany) exercise test following the modified Balke protocol [45] was used to determine the VO_2max. We conducted a warm-up (walking at 3.5 km/h for 1 minute and at 4 km/h for 2 minutes) followed by an incremental protocol which started at a speed of 5.3 km/h at 0% grade for 1 minute. The grade was then increased 1% every minute until the volitional extenuation of the participants was reached. An indirect calorimeter was used to continuously record the gas exchange (VO_2 and VCO_2) using an oronasal mask (model 7400, Hans Rudolph Inc, Kansas City, MO, USA) equipped with a prevent™ metabolic flow sensor (Medgraphics Corp, Minnesota, USA). We performed a flow calibration with a 3-L calibration syringe before the test every day. We calibrated the gas analyser before each test using two standard gas concentrations. The Breeze Suite software (version 8.1.0.54 SP7, MGC Diagnostic®) was used to average VO_2 and VCO_2 every 5 seconds. The 6–20 Borg scale [39] was applied to measure the RPE at each stage and at exhaustion (during the last 15 seconds). A familiarisation process with the RPE scale was conducted before the exercise test. We continuously recorded heart rate values (Polar RS800, Kempele, Finland) every 5 seconds. To reach a RER ≥ 1.1, a plateau in VO_2 (change of <100 ml/min in the last 3 consecutive 10-second stages), and a heart rate between 10 beats/min of the age-predicted maximal heart rate (209–0.73 ∗ age) [46] were established as the criteria for achieving VO_2max. If these criteria were not met, the peak oxygen uptake value during the exercise test was considered [47]. The participants were asked to refrain from stimulant substances 24 hours before the exercise test, to fast for 3 hours, and not to perform any physical activity of moderate (24 hours before) and/or vigorous intensity (48 hours before).

We used a validated isokinetic strength test [48] on a separate day using a Gymnex Iso-2 dynamometer (EASYTECH s.r.l., Italy) and following the same preconditions established in the maximum treadmill test protocol. We performed a concentric test of both knee flexor and extensor muscles at 60° s^{-1}, stabilizing upper members, hips, and shoulders with safety belts. The rotational axis of the dynamometer was aligned with the lateral femoral condyle. We placed the force pad 3–4 cm above the medial malleolus. For safety reasons, we set the knee joint angle between 90° and 170°. We instructed the participants to submaximally flex and extend their knee five times and then to complete three maximal repetitions. A 1-minute rest was established between submaximal and maximal trials [48]. We determined the flexion and extension peak torque as the single repetition with the highest muscular force output (Nm). We counterbalanced the limb order in the test. The participants were strongly motivated during the test.

A digital hand dynamometer (T.K.K. 5401 Grip-D; Takey, Tokyo, Japan) was used to assess hand grip strength (kg). Two attempts were made for each hand, with a 1-minute rest between each trial. We instructed the participants to continuously squeeze for 2-3 seconds and asked them to exert their maximal force in every attempt. Following previous studies, we fixed the grip spam of the dynamometer at 5.5 cm for men and a validated equation was used for women [49]. We considered total hand grip strength as the sum of best attempt on the left and right hand, respectively.

To assess the core strength performance, we conducted the following four endurance tests: (i) the trunk extensor isometric test, (ii) the trunk flexor isometric test, (iii) the side bridge test (which included both left and right sides), and (iv) the front plank test. The participants were given a minimum of 2 minutes between efforts to facilitate recovery. In short, the trunk extensor isometric test was modified from the Biering-Sorensen test [50], which has been previously validated as a reliable measure of back extensor performance [51]. The participants lay prone with the lower body fixed to the test stretcher and keeping their upper bodies on the floor before the exertion. They were instructed to maintain the horizontal position as long as possible, manually recording the endurance time until the upper body came in contact with the floor. The trunk flexor endurance test required the participants to maintain a hip flexion of 60° from the floor, with their knees and hips flexed at 90° [51]. The test ended when the participants were not able to hold the upper body below the 60° angle. The side bridge test consisted of participants lying on an exercise mat on their sides with their legs extended [51]. The participants were instructed to lift their hips off the mat and support themselves on one elbow and their feet. The test ended when the hips touched the exercise mat. The front plank test required the participants to assume a prone position with their shoulders and elbows flexed at 90° [51]. They had to maintain a straight, strong line from head to toes without lowering their hips and keeping their neck in a neutral position with 4 points of support (both forearms and both tiptoes). The test finished when the participants were not able to maintain the correct position.

Statistical analysis

Sample size calculations were based on a minimum predicted 15% change in VO_2max and extension peak torque (with an estimated SD of 15%) between the control group and the exercise groups. Considering the results of a pilot study, 14 individuals per group were necessary to get a statistical power of 85% (type 1 error = 0.05) [52]. Nevertheless, a minimum of 20 participants per group were recruited, since a maximum loss of 25% at follow-up was predicted. Data normality was checked using visual check of histograms, Q-Q plots, and the Shapiro-Wilk test.

A repeated-measures ANOVA was performed to study changes in cardiorespiratory fitness and muscular strength parameters (i.e. VO_2max in absolute and relative terms, maximal heart rate, total test duration, extension peak torque, flexion peak torque, total hand grip, trunk extensor isometric test, side bridge test, and front plank test) across time, between groups, and the interaction (time*group). Student's t tests for paired values were applied to determine intragroup differences in cardiorespiratory fitness and muscular strength parameters before and after the intervention study.

An ANCOVA was performed to study the effect of the groups (fixed factor) on cardiorespiratory fitness and muscular strength parameters, i.e. post-VO_2max minus pre-VO_2max (dependent variable), controlling for the baseline values.

We conducted ANCOVA to analyse the effect of the intervention (group entered as fixed factor) on body composition parameters, i.e. post-VO_2max minus pre-VO_2max (dependent variable), adjusting for the baseline values. The same analyses were conducted for changes in maximal heart rate, total test duration, extension peak torque, flexion peak torque, total hand grip, trunk extensor isometric test, side bridge test, and front plank test. Bonferroni post hoc tests with adjustment for multiple comparisons were used to study changes between all exercise groups.

We fixed the level of significance at $P<0.05$. The Statistical Package for Social Sciences (SPSS, v. 22.0, IBM Corporation, Chicago, IL, USA) was used to conduct the statistical analysis and the GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) to make the graphical plots.

RESULTS

A total of 74 participants (39 women) were included in the analyses after a loss to follow up of 17% (see Figure 1). We registered an attendance of ~99%, ~98%, and ~99% of the supervised exercised sessions in the PAR group, the HIIT group, and the HIIT+EMS group, respectively from week 1 to week 12.

The baseline characteristics of all participants and of each separate group are described in Table 1. No differences were observed in the baseline values between groups.

Figure 2 shows cardiorespiratory fitness-related variables before and after the intervention study. A significant time*group interaction was found in VO_2max in absolute and relative values, and total test duration ($P=0.007$, $P=0.006$, and $P=0.003$, respectively), whereas a near-significant trend toward significance was observed in the time*group interaction in maximal heart rate ($P=0.075$). VO_2max in absolute terms increased in the HIIT group as well as in the HIIT+EMS group (Δ VO_2max=10%; $P=0.033$, and Δ VO_2max=10%; $P<0.001$, respectively). VO_2max in relative terms increased in the PAR group as well as in the HIIT group and in the HIIT+EMS group (Δ VO_2max=11%; $P=0.026$, Δ VO_2max=11%; $P=0.024$, and Δ VO_2max=14%; $P<0.001$, respectively). Total test duration increased in the PAR group as well as in the HIIT group and in the HIIT+EMS group (Δ Total test duration=21%; $P=0.040$, Δ Total test duration=23%; $P=0.003$, and Δ Total test duration=14%; $P=0.006$). No statistical differences were noted in the control group in any case (all $P>0.073$).

A significant time*group interaction was found in extension peak torque, flexion peak torque, and total hand grip ($P<0.001$, $P=0.002$, and $P=0.028$, respectively; Figure 3).

Extension and flexion peak torque increased in the PAR group as well as in the HIIT group and in the HIIT+EMS group (Δ Extension and flexion peak torque=11% and 16% for PAR group, Δ Extension and flexion peak torque=10% and 14% for HIIT group, and Δ Extension and flexion peak torque=23% and 20% for HIIT+EMS group, respectively; all $P \leq 0.003$). Total hand grip increased in the HIIT+EMS group (Δ Total hand grip=7% $P<0.001$). No statistical differences were noted in the control group in any case (all $P>0.270$). A significant time*group interaction was found in the trunk extensor isometric test, trunk flexor isometric test, side bridge test, and front plank test ($P=0.001$, $P<0.001$, $P=0.002$, and $P=0.002$, respectively; Figure 4). The trunk extensor isometric test performance increased in the PAR group as well as in the HIIT group and in the HIIT+EMS group (Δ Trunk extensor isometric test performance=68%; $P<0.001$, Δ Trunk extensor isometric test performance=37%; $P=0.003$, and Δ Trunk extensor isometric test performance=24%; $P=0.050$, respectively). The trunk flexor isometric test performance increased in the HIIT+EMS group (Δ Trunk flexor isometric test performance=20%; $P<0.001$). The side bridge test performance increased in the PAR group as well as in the HIIT group and in the HIIT+EMS group (Δ Side bridge test performance=46%; $P=0.003$, Δ Side bridge test performance=111%; $P<0.001$, and Δ Side bridge test performance=50%; $P<0.001$, respectively). The front plank test performance increased in the PAR group as well as in the HIIT group and in the HIIT+EMS group (Δ Front plank test performance=64% for PAR group, Δ Front plank test performance=79% for HIIT group, and Δ Front plank test performance=64% for HIIT+EMS group; all $P \leq 0.001$).

Figure 5 shows changes in the cardiorespiratory fitness-related variables after the intervention study among the 4 groups. The PAR, HIIT, and HIIT+EMS interventions similarly increased VO_2max in absolute and relative terms, maximal heart rate, and total test duration compared with the control group (all $P \leq 0.034$), with no differences between them (all $P \geq 0.2$). The results persisted in all cases including sex, age, changes in LMI, changes in FMI, changes in energy intake, changes in sedentary time, and changes in overall physical activity levels in the model (see Table 2).

Figure 6 shows changes in muscular strength-related variables after the intervention study among the 4 groups. The PAR, HIIT, and HIIT+EMS interventions similarly improved extension and flexion peak torque and total hand grip compared with the control group (all $P \leq 0.031$), with no differences between them (all $P \geq 0.1$) except when comparing the HIIT vs. the HIIT+EMS group ($P=0.042$).

The results persisted in all cases when sex, age, changes in LMI, changes in FMI, changes in energy intake, changes in sedentary time, and changes in overall physical activity levels were included as a covariate.

Table 1. Descriptive parameters

	All		Control		PAR		HIIT		HIIT+EMS	
	Men (N=35)	Women (N=39)	Men (N=8)	Women (N=12)	Men (N=8)	Women (N=9)	Men (N=9)	Women (N=9)	Men (N=10)	Women (N=9)
Age (years)	54.4 (5.3)	53.0 (5.0)	54.4 (5.3)	53.0 (5.0)	54.5 (5.8)	55.1 (6.1)	52.7 (5.6)	55.8 (4.5)	51.8 (5.4)	51.8 (3.7)
Body composition										
Body mass index (kg/m²)	28.3 (3.6)	25.3 (3.3)	28.3 (3.6)	25.3 (3.3)	28.9 (1.9)	30.5 (3.8)	25.4 (3.1)	25.1 (2.9)	24.0 (2.1)	26.5 (4.7)
Fat mass index (kg/m²)	10.0 (3.2)	11.4 (2.9)	10.0 (3.2)	11.4 (2.9)	10.6 (2.8)	12.0 (3.7)	11.6 (2.6)	11.0 (2.6)	11.0 (2.7)	11.9 (4.1)
Lean mass index (kg/m²)	17.5 (2.0)	13.2 (1.8)	17.5 (2.0)	13.2 (1.8)	17.4 (1.6)	17.6 (2.6)	13.1 (2.5)	13.3 (1.4)	12.4 (1.1)	13.8 (1.6)
Dietary intake										
Total energy (kcal/d)	2271 (437)	1854 (390)	2408 (356)	1694 (338)	2240 (294)	1853 (371)	2384 (493)	1825 (402)	2107 (497)	2021 (446)
Carbohydrate (g/d)	234.5 (64.6)	195.4 (48.3)	265.2 (94.3)	177.1 (60.3)	229.4 (40.2)	190.4 (33.8)	250.1 (56.0)	199.7 (37.0)	201.8 (56.8)	213.2 (60.5)
Fat (g/d)	96.1 (21.4)	78.8 (22.6)	97.1 (11.8)	77.2 (19.3)	97.3 (28.4)	77.3 (24.3)	98.4 (23.7)	72.8 (26.2)	92.3 (21.8)	87.0 (22.1)
Protein (g/d)	89.2 (22.2)	76.7 (26.5)	82.5 (19.0)	63.4 (11.6)	83.7 (12.0)	85.4 (41.3)	96.5 (24.9)	75.3 (22.1)	90.2 (27.2)	79.9 (15.8)
Sedentary behaviour and PA										
Sedentary time (min/day)	770 (80.3)	723.7 (82.6)	768.2 (47.0)	733 (79.5)	757.2 (111.7)	717.2 (101.2)	797.6 (85.9)	725.7 (62.0)	756.6 (69.3)	716.9 (94.8)
LPA (min/d)	169.6 (49.6)	177.8 (40.9)	165.0 (38.5)	174.3 (39.0)	174.3 (70.3)	179.5 (52.5)	163.1 (52.8)	165.0 (32.4)	175.0 (40.1)	191.7 (39.3)
MPA (min/d)	94.3 (34.9)	94.4 (35.3)	97.7 (19.8)	83.4 (32.3)	90.8 (44.8)	100 (46.8)	88.9 (41.6)	90.3 (24.5)	99.7 (32.0)	105.9 (34.8)
VPA (min/d)	2.3 (2.9)	1.1 (1.0)	2.2 (2.4)	0.9 (0.7)	1.4 (1.5)	1.0 (0.7)	3.0 (4.6)	1.4 (1.7)	2.5 (2.5)	1.2 (1.0)
MVPA (min/d)	96.6 (35.5)	95.5 (35.8)	99.9 (18.4)	84.4 (32.8)	92.2 (45.7)	101.0 (47.3)	91.9 (42.1)	91.7 (25.2)	102.2 (33.3)	107.1 (35.4)
Overall PA (ENMO, mG/5 s)	35.8 (8.9)	36.1 (8.8)	36.2 (4.7)	33.8 (8.5)	34.3 (11.8)	37.3 (10.7)	34.9 (10.1)	35.5 (7.9)	37.6 (8.3)	37.9 (8.8)
Cardiorespiratory fitness										
VO_2max (ml/min)	2915 (373)	1809 (332)	2821 (184)	1702 (317)	2795 (494)	1898 (451)	3073 (382)	1850 (283)	2934 (350)	1799 (273)
VO_2max (ml/kg/min)	33.3 (4.5)	27.9 (5.3)	33.1 (3.3)	26.1 (3.7)	35.0 (6.3)	28.7 (4.4)	33.1 (4.6)	30.1 (7.5)	32.2 (3.6)	27.0 (4.9)
Maximal heart rate (b/min)	162.8 (14.6)	160.5 (13.2)	160.3 (17.8)	155.8 (10.6)	163.9 (11.8)	156.3 (16.0)	159.7 (15.9)	166.6 (11.0)	166.4 (14.3)	163.8 (13.4)
Total test duration (s)	828.2 (182.9)	606.5 (164.1)	845.0 (163.1)	554.0 (169.6)	761.9 (215.4)	552.2 (141.7)	802.8 (152.8)	622.2 (117.6)	892.5 (196.6)	703.3 (193.0)
Muscular strength										
Extension peak torque (Nm)	340.2 (67.6)	202.8 (35.4)	314.9 (70.1)	204.3 (39.7)	337.5 (37.7)	212.8 (45.9)	407.7 (52.5)	198.4 (30.3)	297.7 (52.7)	195.3 (24.5)
Flexion peak torque (Nm)	159.1 (43.0)	93.6 (16.6)	146.5 (37.7)	94.7 (17.7)	166.6 (37.8)	95.3 (23.5)	188.1 (49.9)	91.9 (13.6)	134.6 (28.4)	92.2 (11.6)
Total hand grip (kg)	93.1 (12.1)	50.6 (8.2)	91.0 (13.6)	50.8 (8.0)	95.4 (9.8)	51.1 (11.3)	98.2 (11.2)	46.6 (5.0)	88.3 (12.9)	53.9 (6.9)
Trunk extensor isometric test (s)	48.2 (31.2)	52.3 (33.3)	57.4 (45.3)	46.5 (24.5)	43.0 (23.8)	57.2 (44.9)	40.9 (18.4)	51.1 (26.9)	51.7 (34.2)	55.6 (39.6)
Trunk flexor isometric test (s)	157.7 (57.6)	145.2 (59.3)	178.4 (56.9)	155.6 (51.7)	177.1 (44.1)	138.7 (52.8)	133.0 (54.3)	147.5 (57.8)	147.7 (66.5)	136.5 (80.8)
Side bridge test (s)	83.2 (28.3)	61.4 (47.5)	93.0 (15.1)	60.9 (37.7)	88.1 (42.7)	71.4 (81.0)	67.8 (27.0)	41.0 (17.5)	85.6 (19.6)	72.3 (32.9)
Front plank test (s)	56.0 (22.3)	47.0 (26.1)	58.1 (27.1)	49.5 (23.1)	56.4 (25.4)	36.5 (20.8)	54.1 (22.9)	43.5 (22.2)	55.8 (18.4)	57.8 (35.8)

Data are shown as means ± standard deviation. Abbreviations: PAR; Physical Activity Recommendations for adults proposed by the World Health Organization group, HIIT; High Intensity Interval Training group, HIIT+EMS; HIIT plus Whole-Body Electromyostimulation group, PA; Physical Activity, LPA; Light Physical Activity, MPA; Moderate Physical Activity, VPA; Vigorous Physical Activity, MVPA; Moderate-Vigorous Physical Activity.

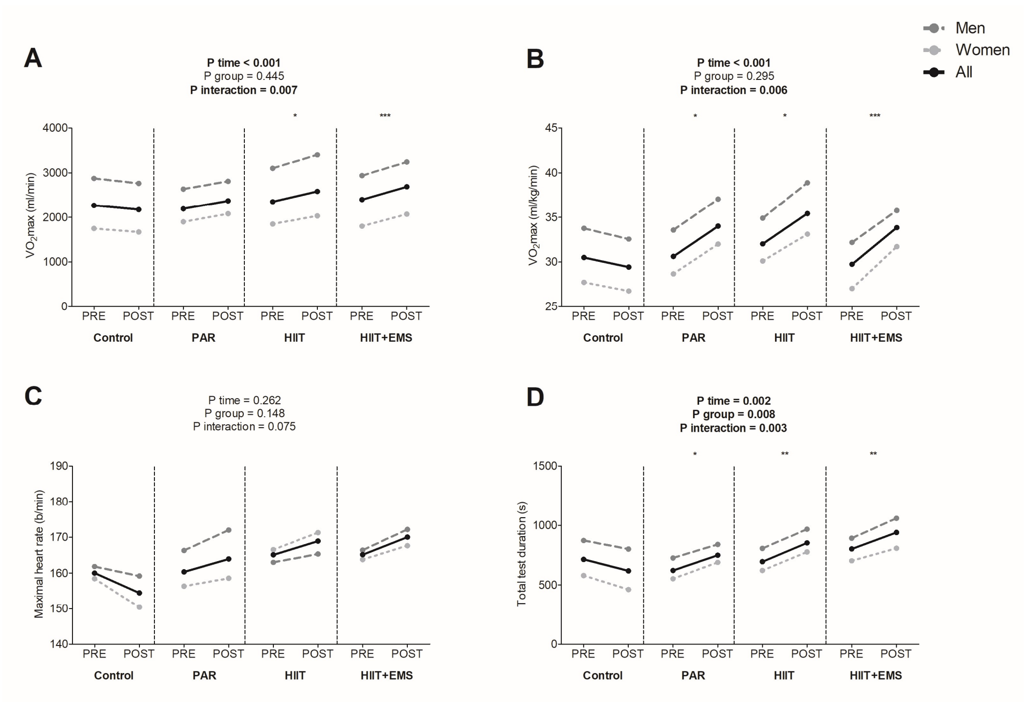

Figure 2. Changes in maximal oxygen uptake (VO2max) in absolute (Figure 2A) and relative terms (Figure 2B), maximal heart rate (Figure 2C) and total test duration (Figure 2D) values before and after the intervention study. P value (time, group, and interaction [time*group]) of repeated measures ANOVA. * P <0.05; ** P < 0.01; *** P < 0.001 obtained by Student's paired t-test. Abbreviations: PAR; Physical Activity Recommendations for adults proposed by the World Health Organization group, HIIT; High Intensity Interval Training group, HIIT+EMS; HIIT plus Whole-Body Electromyostimulation group

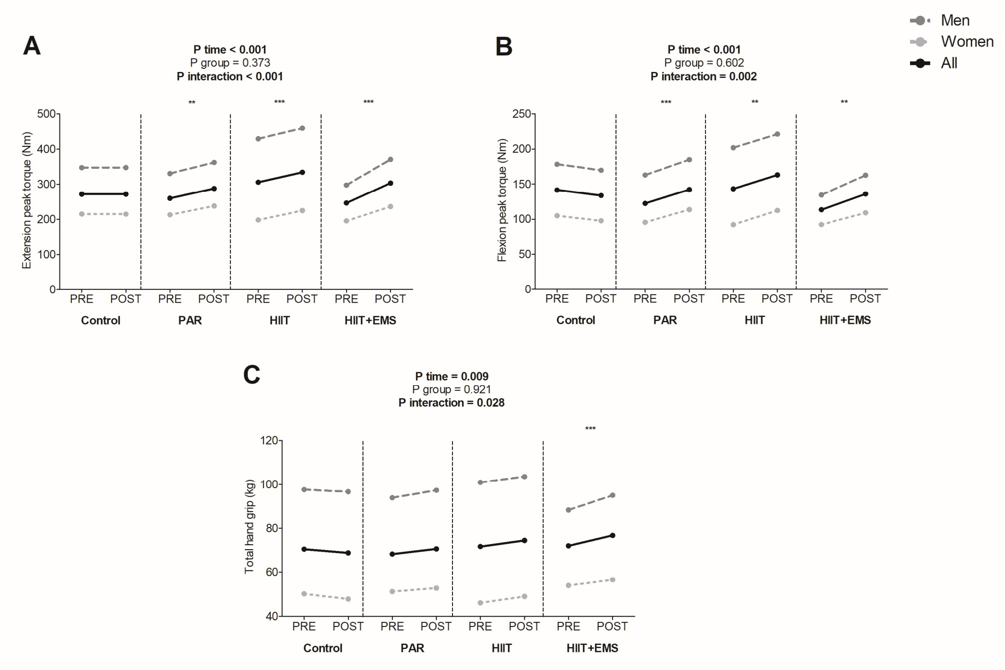

Figure 3. Changes in extension peak torque (Figure 3A), flexion peak torque (Figure 3B), and total hand grip (Figure 3C) values before and after the intervention study. P value (time, group, and interaction [time*group]) of repeated measures ANOVA. * P <0.05; ** P < 0.01; *** P < 0.001 obtained by Student's paired t-test. Abbreviations: PAR; Physical Activity Recommendations for adults proposed by the World Health Organization group, HIIT; High Intensity Interval Training group, HIIT+EMS; HIIT plus Whole-Body Electromyostimulation group.

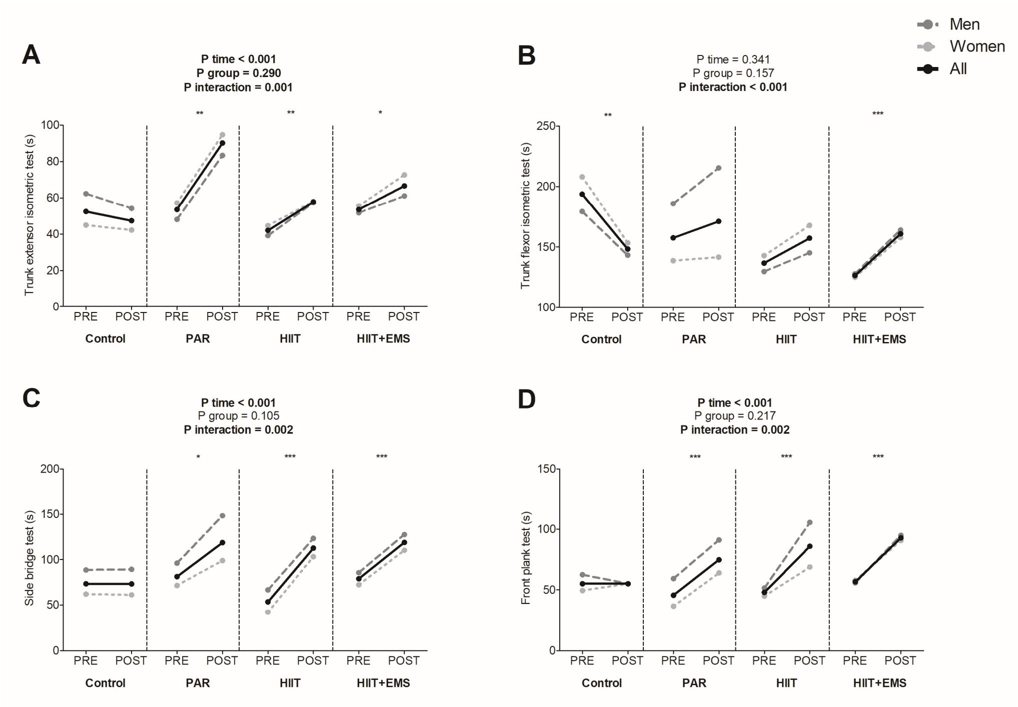

Figure 4. Changes in the trunk extensor isometric test (Figure 4A), trunk flexor isometric test (Figure 4B), side bridge test (Figure 4C), and front plank test (Figure 4D) values before and after the intervention study. P value (time, group, and interaction [time*group]) of repeated measures ANOVA. * P <0.05; ** P < 0.01; *** P < 0.001 obtained by Student's paired t-test. Abbreviations: PAR; Physical Activity Recommendations for adults proposed by the World Health Organization group, HIIT; High Intensity Interval Training group, HIIT+EMS; HIIT plus Whole-Body Electromyostimulation group.

This was an exception for total hand grip, in which we observed a partially attenuated effect including changes in LMI, changes in FMI, changes in energy intake, changes in sedentary time, and changes in overall physical activity levels as a covariate (see Table 2).

Figure 7 shows changes in the core muscular strength-related variables after the intervention study among the 4 groups. The PAR, HIIT, and HIIT+EMS interventions similarly increased the trunk extensor and flexor isometric tests, side bridge test, and front plank test performance compared with the control group (all $P \leq 0.002$). The results persisted when the analyses were additionally adjusted by sex, age, changes in LMI, changes in FMI, changes in energy intake, changes in sedentary time, and changes in overall physical activity (see Table 2).

DISCUSSION

This study shows that a 12-week structured exercise intervention improves physical fitness regardless of the training programme in sedentary middle-aged adults. Despite slightly greater improvements in some fitness variables, the changes observed in the HIIT+EMS group were not superior to the other exercise programmes.

Numerous studies have reported a robust relationship between greater VO_2max and reduced morbidity and mortality risk, which could indicate that the increment of VO_2max observed in our study is a significant and clinically relevant finding [2,3]. Kodama et al. reported that a 1-unit of metabolic equivalents higher level of cardiorespiratory fitness was associated with a decrement of 13% and 15% in risk of all-cause mortality and cardiovascular disease events, respectively, in healthy men and women [2].

In this context, we showed that a 12-week structured exercise intervention increased ~1 metabolic equivalent irrespective of the training programme applied, which is of clinical relevance to quickly and significantly reduce the prevalence of cardiovascular disease events and all-cause mortality.

The absolute increase of VO_2max in the HIIT group concurred with previous studies (~8 to 14%) conducted in similar cohorts [19–21,23]. However, one of these studies compared a 12-week HIIT intervention vs. a 12-week moderate intensity continuous training intervention showing a greater improvement of VO_2max in response to the first one [23].

These results differ from those obtained in our study, since we observed a similar improvement of VO_2max in both the PAR and HIIT groups. This fact could be explained because we combined endurance with resistance training in the PAR group intervention and a recent metanalysis revealed that a well-designed concurrent training programme appears to be beneficial for higher VO_2max physiological adaptations [53].

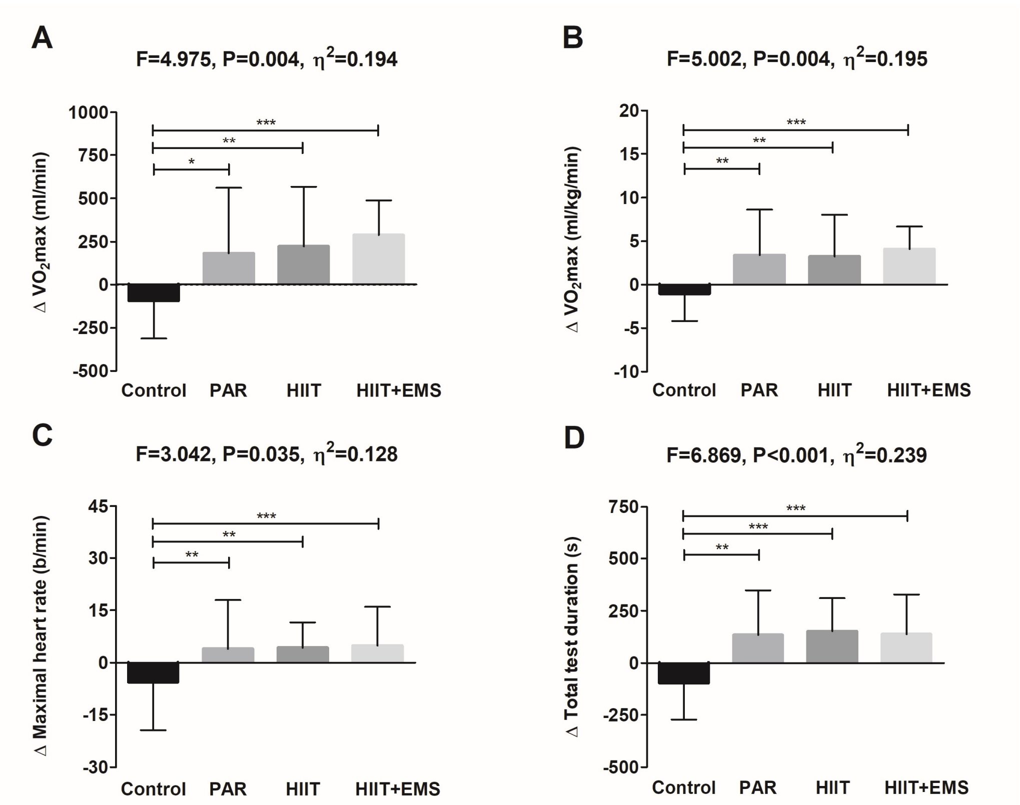

Figure 5. Changes in maximal oxygen uptake (VO_2max) in absolute (Figure 5A) and relative terms (Figure 5B), maximal heart rate (Figure 5C), and total test duration (Figure 5D) after the intervention study among the four groups. Data are shown as means ± standard deviation. Parallel bars indicate significant differences between groups applying an ANCOVA adjusting by baseline values, with post hoc Bonferroni-corrected t-test (*P<0.05; **P<0.01; ***P<0.001). Abbreviations: PAR; Physical Activity Recommendations for adults proposed by the World Health Organization group, HIIT; High Intensity Interval Training group, HIIT+EMS; HIIT plus Whole-Body Electromyostimulation group.

Table 2. Changes in physical fitness outcomes adjusted by baseline values (Model 0), by baseline values and sex (Model 1), by baseline values and age (Model 2), by baseline values and changes in lean mass index (Model 3), by baseline values and changes in fat mass index (Model 4), by baseline values and changes in energy intake (Model 5), by baseline values and changes in sedentary time (Model 6), and baseline values and by changes in overall physical activity levels (Model 7).

	ANCOVA P value							
	Model 0	Model 1	Model 2	Model 3	Model 4	Model 5	Model 6	Model 7
VO_2max (ml/min)	**0.006**	**<0.001**	**0.005**	**0.007**	**0.007**	**0.014**	**0.046**	**0.049**
VO_2max (ml/kg/min)	**0.002**	**0.004**	**0.005**	**0.005**	**0.005**	**0.004**	**0.014**	**0.015**
Maximal heart rate (b/min)	**0.027**	**0.039**	0.053	**0.041**	**0.041**	**0.043**	0.069	0.085
Total test duration (s)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.001**	**0.001**	**0.002**
Extension peak torque (Nm)	**0.001**	**<0.001**	**0.001**	**0.002**	**0.002**	**0.004**	**0.002**	**0.002**
Flexion peak torque (Nm)	**0.001**	**<0.001**	**0.001**	**0.001**	**0.001**	**0.002**	**0.001**	**0.001**
Total hand grip (kg)	**0.031**	**0.003**	**0.027**	0.127	0.130	0.169	0.098	0.082
Trunk extensor isometric test (s)	**0.004**	**0.010**	**0.010**	**0.016**	**0.018**	**0.022**	**0.009**	**0.007**
Trunk flexor isometric test (s)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Side bridge test (s)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Front plank test (s)	**0.003**	**0.004**	**0.007**	**0.001**	**0.002**	**0.016**	**0.004**	**0.005**

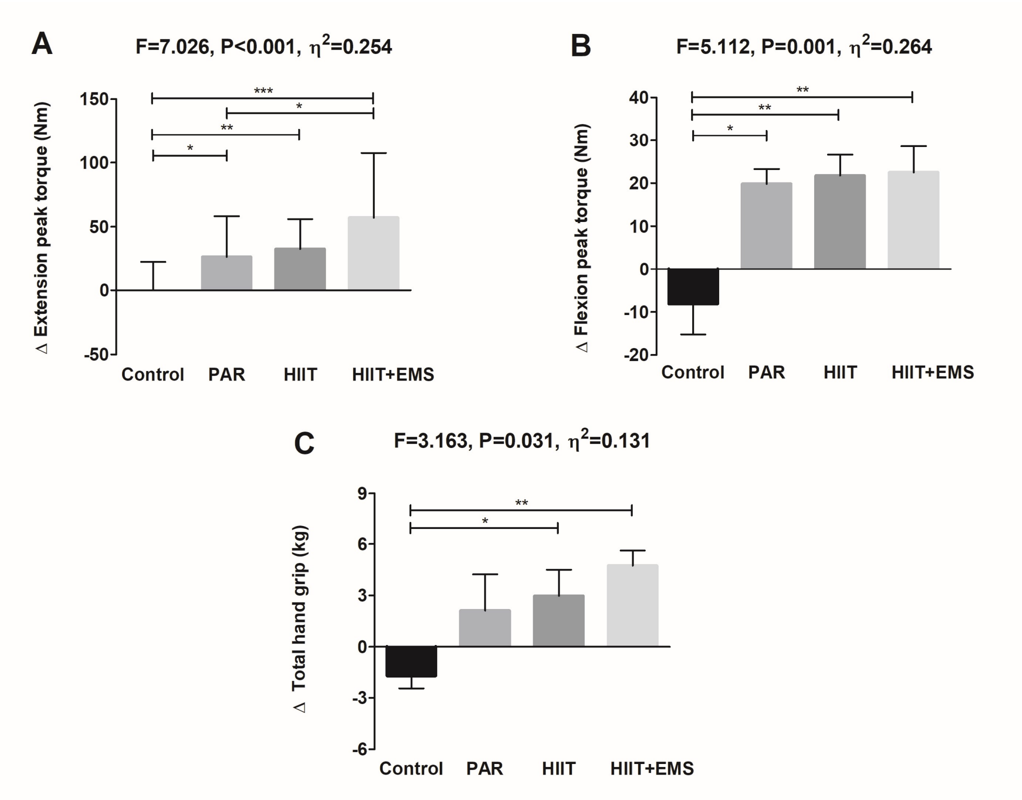

Figure 6. Changes in extension peak torque (Figure 6A), flexion peak torque (Figure 6B), and total hand grip (Figure 6C) after the intervention study among the four groups. Data are shown as means ± standard deviation. Parallel bars indicate significant differences between groups applying an ANCOVA adjusting by baseline values, with post hoc Bonferroni-corrected t-test (*P<0.05; **P<0.01; ***P<0.001). Abbreviations: PAR; Physical Activity Recommendations for adults proposed by the World Health Organization group, HIIT; High Intensity Interval Training group, HIIT+EMS; HIIT plus Whole-Body Electromyostimulation group.

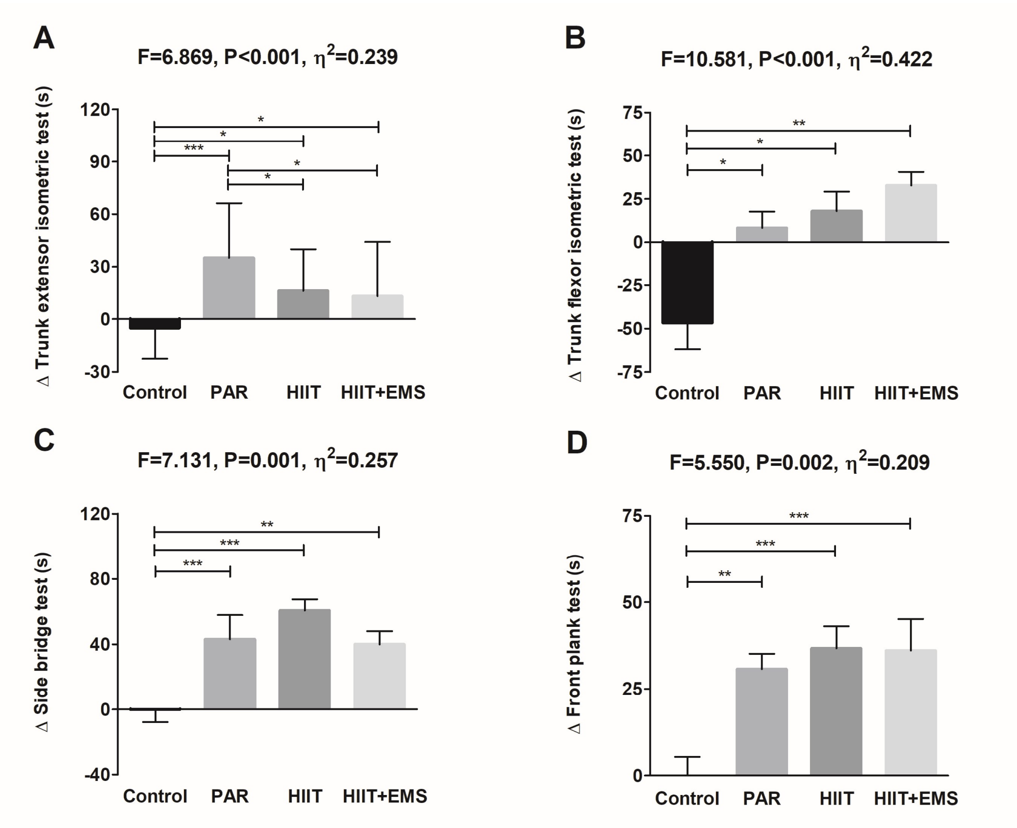

Figure 7. Changes in the trunk extensor isometric test (Figure 7A), trunk flexor isometric test (Figure 7B), side bridge test (Figure 7C), and front plank test (Figure 7D) after the intervention study among the four groups. Data are shown as means ± standard deviation. Parallel bars indicate significant differences between groups applying an ANCOVA adjusting by baseline values, with post hoc Bonferroni-corrected t-test (*P<0.05; **P<0.01; ***P<0.001). Abbreviations: PAR; Physical Activity Recommendations for adults proposed by the World Health Organization group, HIIT; High Intensity Interval Training group, HIIT+EMS; HIIT plus Whole-Body Electromyostimulation group.

Little is known about the effects of WB-EMS on cardiorespiratory fitness. A previous study reported an improvement of VO_2max in healthy adults after a 10-week local electromyostimulation training programme in quadriceps and hamstring muscles [54]. To the best of our knowledge, there is only two study that investigated the influence of WB-EMS on cardiorespiratory fitness suggesting that a 6-week functional and periodised WB-EMS intervention produces an increment of VO_2max (~6%) in trained runners despite a considerable reduction of training volume [24,25]. These findings concur with those obtained in the current study, but it should be noted that we obtained a larger improvement (~13%) as a result of having the longest training programme duration (6 weeks vs. 12 weeks) and having different training status between these two cohorts (trained runners vs. sedentary middle-aged adults). Although some physiological adaptations that could explain an extra VO_2max increment after the application of a WB-EMS programme have been previously described (i.e. [i] a better lower limb coordination and co-activation during exercise, [ii] an increment of the activation capacity of the working muscles during exercise, or [iii] a higher motor unit recruitment and motor unit synchronisation, which may induce better mechanical efficiency and motor recruitment actions [55,56]), no significant improvements in the HIIT+EMS group were noted in our study compared with those obtained in the PAR or the HIIT groups.

It is well-known that muscular strength is negatively and independently associated with all-cause mortality, even controlling by confounder parameters, such as cardiorespiratory fitness, age, or BMI [10,57]. Therefore, to improve muscular strength during the ageing process is of clinical relevance in order to slow down the functional decline and the age-related diseases incidence [1]. A recent systematic review and metanalysis suggested that concurrent training can impact muscular strength to a greater extent than endurance or resistance training alone [53]. Moreover, Sabag et al. highlighted that similar increases in muscular strength and hypertrophy were obtained after a concurrent training programme compared to a HIIT programme including resistance exercise tasks [58]. These findings are consistent with those obtained in our study, since we showed an increase of extension and flexion peak torque and hand grip strength in the PAR group (~10%, ~15%, and 3%, respectively), which concur with the results of previous studies [53,59,60]. The HIIT group also presented a similar magnitude in our study (~9%, ~14%, and 4%, respectively). The effects of WB-EMS on muscular strength have been investigated in previous studies [24,26–31,33]. Their conclusions indicate that this methodology produced significant improvements of: (i) maximum dynamic and isometric leg-press strength in sedentary elderly men (aged >70 years old; ~9%) [28], in elite football players (aged ~25 years old; ~12%) [26], in sedentary elderly women (aged

>70 years old; ~10%) [27], and in postmenopausal sedentary women (aged >70 years old; ~9%) [30]; (ii) vertical jump performance in recreational runners (aged ~27 years old; ~8%) [24], and in elite football players (aged ~25 years old; ~10%) [26]; (iii) maximum hand grip strength in sedentary elderly men (aged >70 years old; ~6%) [29] and in sedentary elderly women (aged >70 years old; ~8%) [31,33]. Our results concur with previous long-term studies, since we showed a significant increase of extension and flexion peak torque and hand grip strength in the HIIT+EMS group (~23%, ~19%, and 6%, respectively). This might be explained because (i) we conducted a functional and periodised HIIT programme adding WB-EMS following the recommendations provided by Filipovic et al. in terms of electrical parameters (impulse frequency, impulse intensity, impulse width, and duty cycle) to effectively improve muscular strength. Most previous studies, however, used a pre-determined training methodology based on isometric weight-bearing exercises (1-2 sets of 8 repetitions) and applied an impulse frequency of 85 Hz, an impulse width of 350 μs, and a duty cycle of 50% [27–33]. (ii) The participant´s characteristics of our study were different than in other studies (i.e. sex, age, training status, etc).

Moreover, although a previous study compared the influence of a HIIT programme vs. a WB-EMS program on cardio-metabolic risk factor in sedentary men [32], there are no studies that compare the effects of these training methodologies on muscular strength in sedentary middle-aged adults applying the same exercises and training loads approach. Our results revealed that, although no significant differences were obtained in muscular strength-related parameters, clinically relevant improvements were noted in the HIIT+EMS group compared to the HIIT group in extension and flexion peak torque and hand grip strength (~23% vs. ~9%; ~19% vs. ~14%; and 6% vs. 4%, respectively). Therefore, our findings suggest that a WB-EMS, as a novel stimulus, could complement the traditional HIIT structure enhancing muscular strength in sedentary middle-aged adults.

Limitations

Our study had a number of limitations. Firstly, the sample size was relatively small to study the influence of these different exercise training interventions on physical fitness considering both sexes separately, although no interaction effects were observed. Considering that we compared a total of three different exercise training programmes, our study could be underpowered to note statistical differences in specific physical fitness-related parameters between them. Moreover, although the results remained after adjusting the analysis for some confounder variables, further trials involving a greater number of participants are needed to accurately determine training induced changes when comparing these three exercise methodologies. Finally, the results of the present study are representative of a sedentary healthy adult population aged between 45 and 65 years old, and therefore might not be extrapolated to active, younger, or older adults, including those with acute or chronic diseases.

CONCLUSIONS

In conclusion, our results suggest that a 12-week structured exercise intervention improves physical fitness regardless of the training programme in sedentary middle-aged adults. Despite slightly greater improvements in some physical fitness variables, the changes observed in the HIIT+EMS group were not superior to the other exercise programmes.

REFERENCES

1 Amaro Gahete FJ, De La O A, Jurado Fasoli L, Castillo MJ, Gutierrez A. Fitness Assessment as an Anti-Aging Marker: A Narrative Review. *J Gerontol Geriatr Res* 2017; **06**.

2 Kodama S, Saito K, Tanaka S, Maki M, Yachi Y, Asumi M *et al.* Cardiorespiratory fitness as a quantitative predictor of all-cause mortality and cardiovascular events in healthy men and women: a meta-analysis. *JAMA* 2009; **301**: 2024–35.

3 Schmid D, Leitzmann MF. Cardiorespiratory fitness as predictor of cancer mortality: a systematic review and meta-analysis. *Ann Oncol* 2015; **26**: 272–278.

4 Artero EG, Lee D, Lavie CJ, España-Romero V, Sui X, Church TS *et al.* Effects of muscular strength on cardiovascular risk factors and prognosis. *J Cardiopulm Rehabil Prev* 2012; **32**: 351–8.

5 Volaklis K, Halle M, Meisinger. Muscular strength as a strong predictor of mortality: A narrative review. *Eur J Intern Med* 2015; **26**: 303–310.

6 Liu Y, Lee D-C, Li Y, Zhu W, Zhang R, Sui X *et al.* Associations of Resistance Exercise with Cardiovascular Disease Morbidity and Mortality. *Med Sci Sports Exerc* 2019; **51**: 499–508.

7 Pedersen B, Saltin B. Exercise as medicine - Evidence for prescribing exercise as therapy in 26 different chronic diseases. *Scand J Med Sci Sport* 2015; **25**: 1–72.

8 Fitzgerald S, Barlow C, Kampert J, Morrow J, Jackson A, Blair S. Muscular fitness and all-cause mortality: prospective observations. *J Phys Act Heal* 2004; **1**: 7–18.

9 Fletcher GF, Landolfo C, Niebauer J, Ozemek C, Arena R, Lavie CJ. Promoting Physical Activity and Exercise: JACC Health Promotion Series. *J Am Coll Cardiol* 2018; **72**: 1622–1639.

10 García-Hermoso A, Cavero-Redondo I, Ramírez-Vélez R, Ruiz JR, Ortega FB, Lee D-C *et al.* Muscular Strength as a Predictor of All-Cause Mortality in an Apparently Healthy Population: A Systematic Review and Meta-Analysis of Data From Approximately 2 Million Men and Women. *Arch Phys Med Rehabil* 2018; **99**: 2100–2113.e5.

11 Harber M, Kaminsky L, Arena R, Blair S, Franklin B, Myers J *et al.* Impact of cardiorespiratory fitness on all-cause and disease-specific mortality: advances since 2009. *Prog Cardiovasc Dis* 2017.

12 Lee, M, Artero, EG, Sui, X, Blair S, Lee D, Artero EG, Sui X, Blair SN. Mortality trends in the general population: the importance of cardiorespiratory fitness. *J Psychopharmacol* 2010; **24**: 27–35.

13 Ozemek C, Laddu DR, Lavie CJ, Claeys H, Kaminsky LA, Ross R *et al.* An Update on the Role of Cardiorespiratory Fitness, Structured Exercise and Lifestyle Physical Activity in Preventing Cardiovascular Disease and Health Risk. *Prog Cardiovasc Dis* 2019; **61**: 484–490.

14 Huang G, Gibson CA, Tran Z V, Osness WH. Controlled endurance exercise training and VO2max changes in older adults: a meta-analysis. *Prev Cardiol* 2005; **8**: 217–25.

15 Liu C-J, Latham NK. Progressive resistance strength training for improving physical function in older adults. *Cochrane database Syst Rev* 2009; **8**: CD002759.

16 WHO | Global recommendations on physical activity for health. *WHO* 2015.

17 Gómez-López M, Gallegos AG, Extremera AB. Perceived barriers by university students in the practice of physical activities. *J Sport Sci Med* 2010; **9**: 374–381.

18 Gibala MJ, Little JP, Macdonald MJ, Hawley JA. Physiological adaptations to low-volume, high-intensity interval training in health and disease. *J Physiol* 2012; **590**: 1077–84.

19 Osawa Y, Azuma K, Tabata S, Katsukawa F, Ishida H, Oguma Y *et al.* Effects of 16-week high-intensity interval training using upper and lower body ergometers on aerobic fitness and morphological changes in healthy men: a preliminary study. *Open access J Sport Med* 2014; **5**: 257–65.

20 Hwang CL, Yoo JK, Kim HK, Hwang MH, Handberg EM, Petersen JW *et al.* Novel all-extremity high-intensity interval training improves aerobic fitness, cardiac function and insulin resistance in healthy older adults. *Exp Gerontol* 2016; **82**: 112–119.

21 Østerås H, Hoff J, Helgerud J. Effects of high-intensity endurance training on maximal oxygen consumption in healthy elderly people. *J Appl Gerontol* 2005; **24**: 377–387.

22 Sculthorpe NF, Herbert P, Grace F. One session of high-intensity interval training (HIIT) every 5 days, improves muscle power but not static balance in lifelong sedentary ageing men A randomized controlled trial. *Med (United States)* 2017; **96**: 1–8.

23 Hurst C, Weston KL, Weston M. The effect of 12 weeks of combined upper- and lower-body high-intensity interval training on muscular and cardiorespiratory fitness in older adults. *Aging Clin Exp Res* 2018; **0**: 0.

24 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.

25 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.

26 Filipovic A, Grau M, Kleinöder H, Zimmer P, Hollmann W, Bloch W. Effects of a Whole-Body Electrostimulation Program on Strength, Sprinting, Jumping, and Kicking Capacity in Elite Soccer

Players. *J Sports Sci Med* 2016; **15**: 639–648.

27 Kemmler W, Bebenek M, Engelke K, von Stengel S. Impact of whole-body electromyostimulation on body composition in elderly women at risk for sarcopenia: the Training and ElectroStimulation Trial (TEST-III). *Age (Dordr)* 2014; **36**: 395–406.

28 Kemmler W, Grimm A, Bebenek M, Kohl M, von Stengel S. Effects of Combined Whole-Body Electromyostimulation and Protein Supplementation on Local and Overall Muscle/Fat Distribution in Older Men with Sarcopenic Obesity: The Randomized Controlled Franconia Sarcopenic Obesity (FranSO) Study. *Calcif Tissue Int* 2018; **103**: 1–12.

29 Kemmler W, Weissenfels A, Teschler M, Willert S, Bebenek M, Shojaa M *et al.* Whole-body electromyostimulation and protein supplementation favorably affect sarcopenic obesity in community-dwelling older men at risk: The randomized controlled FranSO study. *Clin Interv Aging* 2017; **12**: 1503–1513.

30 Kemmler, Schliffka R, Mayhew JLJ, von Stengel S, Schliffka S, Mayhew JLJ *et al.* Effects of whole-body electromyostimulation on resting metabolic rate, body composition, and maximum strength in postmenopausal women: the Training and ElectroStimulation Trial. *J Strength Cond Res* 2010; **24**: 1880–7.

31 Kemmler, Teschler M, Weissenfels A, Bebenek M, von Stengel S, Kohl M *et al.* Whole-body electromyostimulation to fight sarcopenic obesity in community-dwelling older women at risk. Results of the randomized controlled FORMOsA-sarcopenic obesity study. *Osteoporos Int* 2016; **27**: 3261–3270.

32 Kemmler, Teschler M, Weißenfels A, Bebenek M, Fröhlich M, Kohl M *et al.* Effects of Whole-Body Electromyostimulation versus High-Intensity Resistance Exercise on Body Composition and Strength: A Randomized Controlled Study. *Evidence-based Complement Altern Med* 2016; **3**: 44–55.

33 Von Stengel S, Bebenek M, Engelke K, Kemmler W. Whole-Body Electromyostimulation to Fight Osteopenia in Elderly Females: The Randomized Controlled Training and Electrostimulation Trial (TEST-III). *J Osteoporos* 2015; **11**: 1–7.

34 Schulz KF, Altman DG, Moher D, Jüni P, Altman D, Egger M *et al.* CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMC Med* 2010; **8**: 18.

35 Schulz KF, Grimes DA. Generation of allocation sequences in randomised trials: chance, not choice. *Lancet (London, England)* 2002; **359**: 515–9.

36 Storlien L, Oakes ND, Kelley DE. Metabolic flexibility. *Proc Nutr Soc* 2004; **63**: 363–8.

37 Buchheit M, Laursen PB. High-intensity interval training, solutions to the

programming puzzle: Part II: Anaerobic energy, neuromuscular load and practical applications. *Sport Med* 2013; **43**: 927–954.

38 Buchheit M, Laursen PB. High-intensity interval training, solutions to the programming puzzle: Part I: Cardiopulmonary emphasis. *Sport Med* 2013; **43**: 313–338.

39 Borg GA. Psychophysical bases of perceived exertion. *Med Sci Sports Exerc* 1982; **14**: 377–81.

40 Amaro-Gahete FJ, de la O A, Jurado-Fasoli L, Ruiz JR, Gutiérrez Á. 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.

41 Baugh E, Hedrick VE, Marinik EL, Kyle D. Dietary Intake Modification in Response to Participation in a Resistance Training Program for Sedentary Older Adults with Prediabetes: Findings from the Resist Diabetes Study. *Eat Behav* 2015; **15**: 379–382.

42 López, M. D. R., Martín-Lagos RA. *Guía para estudios dietéticos: álbum fotográfico de alimentos*. Editorial Universidad de Granada, 2010.

43 Hildebrand M, Hansen BH, van Hees VT, Ekelund U. Evaluation of raw acceleration sedentary thresholds in children and adults. *Scand J Med Sci Sport* 2017; **27**: 1814–1823.

44 Hildebrand M, Van Hees VT, Hansen BH, Ekelund U. Age group comparability of raw accelerometer output from wrist-and hip-worn monitors. *Med Sci Sports Exerc* 2014; **46**: 1816–1824.

45 Balke B, Ware RW. An experimental study of physical fitness of Air Force personnel. *U S Armed Forces Med J* 1959; **10**: 675–88.

46 Tanaka H, Monahan K, Seals D. Age-predicted maximal heart rate revisited. *J Am Coll Cardiol* 2001; **37**: 153–6.

47 Midgley AW, McNaughton LR, Polman R, Marchant D. Criteria for determination of maximal oxygen uptake: A brief critique and recommendations for future research. *Sport Med* 2007; **37**: 1019–1028.

48 Artero, EG, Espada-Fuentes, JC, Argüelles-Cienfuegos, J, Román, A, Gómez-López, PJ, Gutiérrez A. Effects of whole-body vibration and resistance training on knee extensors muscular performance. *Eur J Appl Physiol* 2012; **112**: 1371–1378.

49 Ruiz-Ruiz J, Mesa J, Gutiérrez A, Castillo M. Hand size influences optimal grip span in women but not in men. *J Hand Surg Am* 2002; **27**: 897–901.

50 Biering-Sørensen F. Physical measurements as risk indicators for low-back trouble over a one-year period. *Spine (Phila Pa 1976)* 1984; **9**: 106–19.

51 McGill, S, Childs, A, Liebenson C. Endurance times for low back stabilization exercises: clinical targets for testing and training from a normal database. *Arch Phys Med Rehabil* 1999; **80**: 941–944.

52 Shaffer ML, Chinchilli VM. Including

multiple imputation in a sensitivity analysis for clinical trials with treatment failures. *Contemp Clin Trials* 2007; **28**: 130–137.

53 Murlasits Z, Kneffel Z, Thalib L. The physiological effects of concurrent strength and endurance training sequence: A systematic review and meta-analysis. *J Sports Sci* 2018; **36**: 1212–1219.

54 Nuhr M, Crevenna R, Gohlsch B, Bittner C, Pleiner J, Wiesinger G *et al.* Functional and biochemical properties of chronically stimulated human skeletal muscle. *Eur J Appl Physiol* 2003; **89**: 202–8.

55 Filipovic A, Kleinöder H, Dörmann U, Mester J. Electromyostimulation--a systematic review of the effects of different electromyostimulation methods on selected strength parameters in trained and elite athletes. *J strength Cond Res* 2012; **26**: 2600–14.

56 Filipovic A, Kleinöder H, Dörmann U, Mester J. Electromyostimulation--a systematic review of the influence of training regimens and stimulation parameters on effectiveness in electromyostimulation training of selected strength parameters. *J strength Cond Res* 2011; **25**: 3218–38.

57 Ruiz JR, Sui X, Lobelo F, Morrow JR, Jackson AW, Sjöström M *et al.* Association between muscular strength and mortality in men: prospective cohort study. *BMJ* 2008; **337**: a439.

58 Sabag A, Najafi A, Michael S, Esgin T, Halaki M, Hackett D. The compatibility of concurrent high intensity interval training and resistance training for muscular strength and hypertrophy: a systematic review and meta-analysis. *J Sports Sci* 2018; **36**: 2472–2483.

59 Takeshima N, Rogers ME, Islam MM, Yamauchi T, Watanabe E, Okada A. Effect of concurrent aerobic and resistance circuit exercise training on fitness in older adults. *Eur J Appl Physiol* 2004; **93**: 173–182.

60 Eklund D, Häkkinen A, Laukkanen JA, Balandzic M, Nyman K, Häkkinen K. Fitness, body composition and blood lipids following 3 concurrent strength and endurance training modes. *Appl Physiol Nutr Metab* 2016; **41**: 767–774.

Chapter 12:
Basal metabolic rate and fat oxidation in basal conditions and during exercise in sedentary middle-aged adults, following different exercise training interventions: a randomised controlled trial. THE FIT-AGEING Study **(Study 16)**

ABSTRACT

This study compares the influence of different exercise training programs on BMR, BFox and MFO, in sedentary, middle-aged adults. The study subjects of this 12 week-long, randomised, controlled trial, were 71 middle-aged adults (age 53.5±4.9 years; 52% women). Subjects were randomly assigned to one of the following groups: (1) no exercise, (2) PAR group, (3) HIIT group, and (4) HIIT+EMS group. Subject BMR, BFox and MFO were determined by indirect calorimetry before and after the intervention. The HIIT+EMS subjects showed significant increases in BMR and BFox following the intervention (all $P<0.03$); no such differences were seen in the PAR, HIIT or control groups (all $P\geq0.1$). A significant increase in post-intervention MFO was noted for the HIIT+EMS group compared to the non-exercise control group ($P<0.01$); no such difference was seen in the PAR or HIIT groups compared to the control group (all $P\geq0.05$).

Twelve weeks of high intensity interval training plus whole-body electromyostimulation increased the BMR, BFox and MFO of middle-aged sedentary adults. These findings have important clinical implications; a well-designed high-intensity interval training program plus whole-body electromyostimulation might be followed to help combat the appearance of chronic metabolic diseases characterized by metabolic inflexibility in middle-aged sedentary adults, though it will be necessary to determine how long the effects last.

BACKGROUND

The BMR accounts for a large part (60-70%) of total energy expenditure [1,2]. Under basal conditions, the human body derives more than half of its energy from the oxidative metabolism of fatty acids; the remainder is mainly derived from glucose [3]. The proportion and quantity of the different nutrients oxidised can be estimated by IC and the use of stoichiometric equations [4].

Metabolic flexibility is defined as the ability to adapt energy requirements and fuel oxidation to fuel availability and environmental demands [5]. The ability to increase fat oxidation under basal conditions has traditionally been regarded a powerful indicator of metabolic flexibility [6]. While metabolic flexibility has been amply studied under post-fast, post-prandial, and hyperinsulinaemic euglycaemic clamp conditions, its relationship with exercise and training has been much less explored [5]. MFO and Fat_{max} have been proposed key indicators of metabolic flexibility during exercise [5,7]. The development of strategies (i.e., dietary or physical exercise interventions) aimed at increasing metabolic flexibility may offer a means of combating excessive fat accumulation and obesity. Physical exercise significantly increases (i) skeletal muscle mitochondrial biogenesis [8], (ii) mitochondrial activity [8], and (iii) fatty acid oxidation capacity [9,10], improving metabolic flexibility [5,8].

International physical activity guidelines suggest that 150-300 min/week of moderate-vigorous physical activity combined with resistance training 2 days/week are enough to obtain health benefits [11,12]. However, the lack of time in developed societies makes it hard to adhere to such recommendations. In general, <5% of the population may undertake 30 min/day of objectively measurable physical activity [13,14]. Time-efficient exercise training modalities have thus been developed, and low-volume HIIT has been reported as good a stimulus - or even better - than continuous moderate intensity training in terms of cardiorespiratory fitness and body composition [15,16]. Relatively little attention has been paid, however, to the influence of this type of exercise on BMR, BFox and/or MFO.

WB-EMS, a recently emerged exercise training methodology, combines simultaneous active and passive muscle contractions via exercise and the electrical stimulation of skeletal muscle groups [17]. Several studies have examined the effect of WB-EMS on physical fitness [18–22] and/or body composition [20,23–28], and generally suggest it to be associated with an increase in LM in sedentary and moderately trained young, middle-aged and elderly individuals [20,23–28]. It is therefore plausible that such training could influence BMR, BFox, and MFO. To our knowledge, only one study has studied the effects of a 14-week WB-EMS program on BMR in moderately-trained post-menopausal women [20], with significant improvements in body composition and muscular strength detected, but no significant increase in BMR [20]. It remains unknown, however, whether these findings apply to sedentary individuals of either sex. Moreover, there have been no studies comparing the effect of different exercise training programs on BMR, BFox, and MFO in sedentary middle-aged adults.

The present work study compares the influence of different exercise training programs - no exercise, PAR [11,12], HIIT, and HIIT+EMS- on BMR, BFox, and MFO in sedentary middle-aged adults. Moreover, we also studied the predictors of BMR, BFox, and MFO in sedentary middle-aged adults.

MATERIAL & METHODS

This manuscript adheres to the CONSORT statement for improving the reporting of parallel group randomised trials (available at EQUATOR Network: *http://www.equator-network.org/reporting-guidelines/consort/*) [29].

Setting and eligibility criteria

Eighty-nine sedentary, middle-aged adults (37 women) aged 45-65 years whose weight was stable over the previous three months, were recruited to participate in the current study (clinicaltrial.gov: ID: NCT03334357) [30]. 'Sedentary' was defined as performing <20 min of moderate-intensity physical activity on 3 days/week over the previous three months (self-reported). All subjects confirmed being free of cardiovascular disease, diabetes mellitus, cancer, and any disease associated with exercise intolerance. This study was approved by the Human Research Ethics Committee of the *Junta de Andalucía* [0838-N-2017] and complied with the latest revision of the Declaration of Helsinki. All subjects provided their written informed consent to be included after receiving detailed oral and written information on the study procedures.

Procedures

Social networks, local media and posters were used to recruit study subjects. Those deemed potentially eligible were contacted by telephone or e-mail and invited to attend an interview. The baseline examination involved two assessment days. On day 1, BMR and basal fuel oxidation were assessed, followed by a graded exercise test to determine the MFO and Fat_{max}. On day 2 (between 3 and 5 days after day 1), a maximum effort test was conducted to determine VO_2max.

Interventions

The present study was designed as a 12-week randomised controlled trial with parallel groups. For practical and feasibility reasons it was conducted in two waves (September-December 2016, and September-December 2017). After the baseline evaluation, the participants were randomly allocated to one of four exercise programs using a computer-generated simple randomisation procedure [31]:

(1) no exercise (control group), (2) PAR group, (3) HIIT group, and (4) HIIT+EMS group. The exercise training program descriptions adhere to the Consensus on Exercise Reporting Template (CERT) [32], increasing the transparency and replicability of this work. A methodological manuscript explaining all three exercise training programs is available elsewhere [30].

Attendance at the training sessions (described below) was recorded daily; subjects who missed a session were asked the reason for their absence and requested to make up for it on another day in the same week. A minimum 90% attendance rate was deemed necessary for valid data to be extracted. No home-based sessions were programmed. Sessions were performed in groups of 2-6 subjects, allowing their safety to be monitored and ensuring that all training volume and intensity requirements were met. The exercise training sessions included a standardised warm-up and cooling-down protocol (see methodological manuscript [30]).

The PAR subjects performed three sessions per week (i.e., 36 sessions in total) of concurrent training. All completed 150 min per week at 60-65% of the HRres in aerobic training, plus 60 min per week at 40-50% of 1RM of resistance training. The required aerobic training was performed in 10 min bouts using different ergometers (treadmill, cycle-ergometer and elliptical ergometer). The required resistance training included global strength training exercises (½ squat, Romanian deadlift, bench press, and lateral pull-down, among other) using weight-bearing and pneumatic machines. A recovery period of at least 48 h was allowed between the exercise training sessions.

The HIIT subjects performed two sessions per week (i.e., 24 sessions in total) of two HIIT protocols, i.e., a long intervals protocol on day 1, and a short intervals protocol on day 2 [33,34]. The training volume was set to 40-65 min per week at an intensity of >95% of VO_2max in HIIT with long intervals, and at a value of 6-9 on a RPE scale [35] in HIIT with short intervals. The subjects walked on a personalised slope adapted to the fixed intensity of the HIIT with short intervals exercise training sessions, while a weight-bearing training circuit (i.e., dead lift, horizontal row, high heels up, frontal plank, push up, lateral plank, squat, and high knees up) was designed for the HIIT with short intervals training sessions.

The HIIT+EMS subjects performed an exercise training program with the same structure as the HIIT group, i.e., the same volume, intensity, frequency, type of exercise, training load variation, training periodisation, and training sessions, but with the inclusion of electrical impulses. Since the subjects had never been exposed to WB-EMS, a progressive, gradual training period was designed requiring (i) rectangular, bipolar, and symmetrical electric pulses at an intensity of ~100 mA in HIIT with long intervals sessions, and ~80 mA in HIIT with short intervals sessions, (ii) a duty cycle of ~100% in HIIT with long intervals sessions and ranging from 50 to 63% in HIIT with short intervals sessions, (iii) an impulse width ranging from 200 to 400 µs, and (iv) a frequency range of 15 to 20 Hz in HIIT with long intervals sessions, and 35 - 75 Hz in HIIT with short intervals sessions, using the WB-EMS device manufactured by Wiemspro® (Malaga, Spain).

Control group

The subjects assigned to the control group were provided with general advice on a healthy lifestyle, including nutritional information and physical activity guidelines.

Outcome measures

BMR and fuel oxidation in basal conditions

BMR was measured in the morning after a 12 h overnight fast. The participants were instructed to arrive at the laboratory in a motor vehicle to avoid exertion. The evening meal of the previous day was standardised: an egg omelette plus boiled rice and tomato purée. Subjects were asked to avoid any moderate or vigorous physical activity before the test day for 24 h and 48 h respectively, and to sleep as usual. The assessments were conducted in a quiet, mildly light room, with controlled environmental conditions (temperature 22-24ºC, humidity 35-45%). Upon arrival, subjects were required to lie on bed in a supine position for at least 15 min before starting the BMR test, which lasted 30 min [36,37]. VO_2 and VCO_2 were measured by IC using an Ultima CardiO_2 metabolic cart (Medgraphics Corp, Minnesota, USA), and employing a neoprene face-mask without external ventilation [38]. Prior to the beginning of BMR measurement, two standard gas concentrations were used to calibrate the gas analyzer following the manufacturer's recommendations. A 3 L calibration syringe was used to calibrate the turbine ventilometer. Subjects were asked not to fidget, talk or sleep, and to breath normally.

For the calculation of the BMR, Breeze Suite software v.8.1.0.54 was used to average the ventilatory variables every 1 min. The first 5 min-worth of data were discarded, and the CVs for the VO_2, VCO_2, RER, and minute ventilation then determined for every 5 min period [36,37]. The periods that met the steady state criteria for the RER (CV <10% for VO_2, CV <10% for VCO_2, CV <5%, and for minute ventilation (CV <10%) were then selected, and the period with the lowest average CV for the VO_2, VCO_2, RER and minute ventilation chosen for further analysis [37]. Subjects with a RER of <0.7 or >1.0 were excluded. BMR and BFox were calculated using the stoichiometry equations of Weir [39] and Frayn [4] respectively. BMR was expressed in absolute term (kcal/day) and relative to the LM (kcal/$kg_{leanmass}$/day). BFox was expressed in absolute term (g/min) and as a percentage of BMR.

MFO and Fat_{max}

MFO and FAT_{max} were assessed via a submaximal graded exercise test using an H/P/Cosmos Pulsar treadmill (H/P/Cosmos Sports & Medical GmbH, Nussdorf-Traunstein, Germany) [40,41]. After the determination of the maximum walking speed, a warm-up at 3.5 km/h (gradient 0%) was allowed. The treadmill speed was then increased by 1 km/h every 3 min until the maximum walking speed was reached. The gradient was then increased by 2% every 3 min, keeping the treadmill speed constant. The submaximal graded exercise test finished when a RER of >1.0 was reached [42]. VO_2 and VCO_2 were obtained by IC throughout the exercise, using an Ultima CardiO2 metabolic cart (Medgraphics Corp, Minnesota, USA), calibrated as explained above, and employing a Model 7400 face mask (Hans Rudolph Inc, Kansas City, MO, USA), equipped with a prevent™ metabolic flow sensor (Medgraphics Corp, Minnesota, USA) for gas data collection. Breeze Suite software v.8.1.0.54 was used to average the ventilatory variables every 10 s. Fat oxidation data was estimated from the VO_2 and VCO_2 values averaged over the final 1 min of each 3 min stage [43] using the Frayn stoichiometric equation, assuming urinary nitrogen excretion to be negligible [4]. To estimate the MFO and Fat_{max} for each subject, a third-degree polynomial regression curve was constructed with an intersection at 0;0, plotting the fat oxidation data obtained in the submaximal graded exercise test against the relative exercise intensity (expressed as a percentage of VO_2max) [43]. MFO was expressed in absolute term (g/min) and relative to the LM (mg/$kg_{leanmass}$/min).

Anthropometry and body composition

Weight (kg) and height (cm) were measured using a Seca Model 799 electronic scale and stadiometer (Seca, Hamburg, Germany). The BMI was determined as *weight (kg)/height (m)*^{2}. Body composition was assessed using a Discovery Wi dual-energy X-ray absorptiometer (Hologic, Inc., Bedford, MA, USA), obtaining FM and LM following the manufacturer's recommendations.

VO_2max

VO_2max was determined on a separate day via a maximum effort test following a modified version of the Balke protocol [44]. VO_2 and VCO_2 were also measured via IC, gathering data as for MFO and Fat_{max} testing (see above).

Data for a number of blood analytical variables, physical activity, sedentary time, and dietary intakes were also collected.

Statistical analysis

The normal distribution of the main variables was confirmed by histograms and Q-Q plots. Sample size was determined based on a pilot study [30]. Descriptive characteristics are presented as means ± SDs. Differences in the baseline characteristic between the different groups were sought with ANOVA. Given the aim of assessing the efficacy of the exercise training interventions with respect to the outcome variables, primary analysis was performed per-protocol, including only those subjects who completed the exercise training programs and the post-test evaluation. A sensitivity analysis (BOCF imputation) was performed to check the robustness of the results.

Repeated-measures ANOVA was used to detect changes in BMR, BFox, MFO, and Fat_{max} over time, between groups, and to assess the influence of the interaction *time x group*. The Student paired t test was used to examine differences in dependent outcome variables within groups before and after the intervention.

ANCOVA was performed to compare the changes in the BMR (e.g., post-BMR minus pre-BMR [dependent variable]) between groups (fixed factor), adjusting for the post-fast baseline values. Similar analyses were performed for changes in BFox, MFO, and Fat_{max}. Bonferroni post hoc adjustment for multiple comparisons was used to examine the changes between all exercise types. The same analyses were also conducted controlling for confounders (age, and sex).

Simple and multiple linear regression was used to study the relationships between changes in body composition, blood variables, physical activity and sedentary time variables, dietary intake and macronutrient distribution, and cardiorespiratory fitness, with changes in BMR, BFox, and MFO, adjusting for age and sex.

All calculations were made using the Statistical Package for the Social Sciences v.22.0, (IBM Corporation, Chicago, IL, USA). Graphs were plotted using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Significance was set at $P<0.05$.

RESULTS

Figure 1 shows the trial flowchart. No adverse events were recorded during the exercise sessions. No significant difference between the groups was detected for any variable at baseline (all $P \geq 0.068$, Table 1).

Figure 2 shows the changes in BMR and BFox at the end of the intervention. The interaction *time x group* had no influence on BMR and on BFox (all $P \geq 0.1$, Figure 2).

Comparing within-group changes, the HIIT+EMS group showed significantly larger changes in BMR, and in BFox (expressed in g/min and as %BMR), after the intervention (1511.3±69.5 vs. 1653.5±80.1 kcal/day, $P=0.030$, Figure 2A; 0.051±0.007 vs. 0.068±0.001 g/min, $P=0.050$, Figure 2E; and 43.5±6.3 vs. 56.7±6.3 % of BMR, $P=0.010$ Figure 2G, respectively). No significant changes were seen, however, in the PAR, HIIT or control groups (all $P \geq 0.1$). ANCOVA, adjusting for baseline values, revealed no significant differences between groups in terms of the change in BMR, nor in terms of the change in BFox (all $P \geq 0.140$, Figure 2B, 2D, 2F). However, a strong trend towards significance was seen between groups with respect to the change in BFox when expressed as %BMR ($P=0.059$). Bonferroni post-hoc correction revealed a significant increase in BFox in the HIIT+EMS group compared to the control group (+7.0±4.7 vs. -6.5±5.4 % of the BMR, $P=0.043$, Figure 2H), and a near-significant trend in the change in BMR was noted in the HIIT+EMS group compared to the control group (140.0±45.5 vs. -35.5±77.1 kcal/day, $P=0.087$, Figure 2B). These results were consistent across intention to treat sensitivity analyses (data not shown). All findings persisted after controlling for sex and age (see Table 2).

Figure 3 shows the changes in MFO (ΔMFO), and Fat_{max} at the end of the intervention period. The interaction *time x group* had a significant influence on ΔMFO ($P=0.009$, Figure 3A), but none on ΔMFO expressed relative to LM, nor on the change in Fat_{max} (all $P\geq0.7$, Figure 3C and 3E).

When comparing within-group changes, the HIIT+EMS group showed a significantly larger increase in MFO at the end of the intervention (0.29±0.02 vs. 0.33±0.02 g/min, Figure 3A, $P=0.008$), whereas no significant change was detected for the PAR, HIIT or control groups (all $P\geq0.05$). ANCOVA, adjusting for baseline values, revealed significant differences between groups in terms of ΔMFO ($P=0.034$, Figure 3B), whereas no significant differences were detected in ΔMFO when expressed relative to LM or Fat_{max} (all $P\geq0.5$, Figure 3D and 3F). Bonferroni post hoc correction indicated the MFO to be significantly increased in the HIIT+EMS and HIIT groups compared to the control group (+0.05±0.02 and +0.03±0.02 vs. -0.01±0.02 g/min, $P=0.002$ and $P=0.033$, respectively, Figure 3B). All of these findings persisted after controlling for sex and age (see Table 2), and all results were consistent across intention to treat sensitivity analyses (data not shown). No significant associations were observed between changes in body composition variables, blood parameters, physical activity and sedentary time, dietary intake or cardiorespiratory fitness, and changes in BMR (all $P\geq0.05$, Table 3). However, an association approaching significance was noted between changes in energy intake and changes in BMR ($\beta=-0.076$, $R^2=0.074$, $P=0.051$; Table 4), which remained after controlling for age and sex. Significant associations were found between the changes in FM and LM, and changes in BFox ($\beta=-0.002$, $R^2=0.163$, respectively; Table 5), which persisted after including sex and age in the models. Significant associations were noted between cardiorespiratory fitness (both in terms of absolute values and when expressed relative to weight) and ΔMFO ($\beta=-0.470$, $R^2=0.221$, $P<0.001$ and $\beta=0.006$, $R^2=0.197$, $P=0.001$, respectively; Table 4), which remained after adjusting for sex and age.

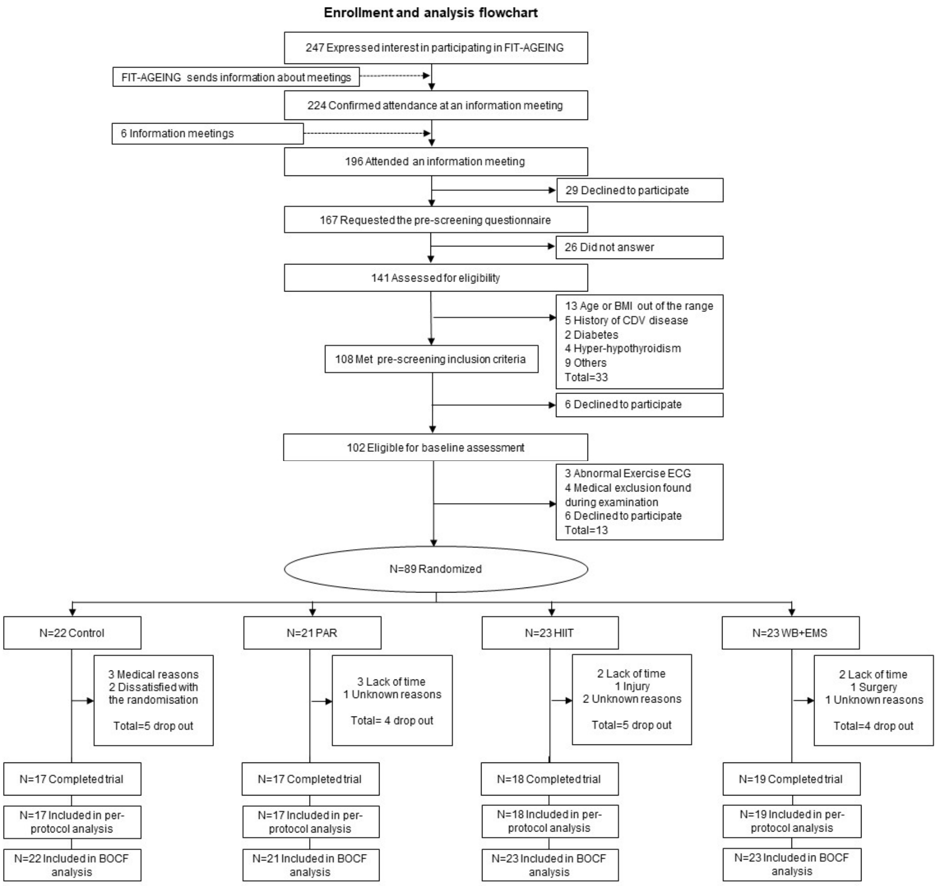

Figure 1: Recruitment and analysis flow-chart. Abbreviations: BMI body mass index, CDV cardiovascular, ECG electrocardiogram, PAR physical activity recommendations group, HIIT high intensity interval training group, HIIT+EMS HIIT plus whole-body electromyostimulation group, BOCF baseline observation carried forward imputation.

Table 1. Baseline descriptive characteristics of the study subjects included in the per-protocol analysis.

	All (n=71)	Control (n=17)	PAR (n=17)	HIIT (n=18)	HIIT+EMS (n=19)	P value
Age (years)	53.4 (4.9)	52.1 (4.1)	54.9 (4.5)	53.1 (5.6)	53.5 (5.3)	0.414
Sex (%)						
Men	34 (47.9)	7 (41.2)	8 (47.1)	9 (50)	10 (52.6)	0.921
Women	37 (52.1)	10 (58.8)	9 (52.9)	9 (50)	9 (47.4)	
Body composition						
BMI (kg/m²)	26.82 (3.79)	26.67 (3.71)	25.41 (2.86)	26.43 (3.15)	28.60 (4.64)	0.077
FM (kg)	30.15 (8.39)	28.64 (6.85)	26.83 (6.31)	31.42 (8.30)	33.27 (10.36)	0.786
LM (kg)	43.92 (11.59)	42.92 (12.06)	43.60 (10.77)	44.43 (13.52)	44.60 (10.76)	0.972
Blood variables						
Plasma glucose (mg/dL)	93.56 (11.36)	93.47 (10.82)	93.35 (11.63)	90.06 (5.56)	96.95 (14.80)	0.352
Plasma insulin (uUI/mL)	8.08 (5.68)	7.26 (5.05)	7.52 (3.97)	7.09 (4.51)	10.22 (7.88)	0.296
HOMA index	1.93 (1.67)	1.73 (1.37)	1.75 (0.99)	1.59 (1.05)	2.59 (2.55)	0.255
Physical activity and sedentary time						
Sedentary time (min/day)	746.93 (84.34)	751.19 (68.78)	736.02 (104.87)	763.77 (82.10)	737.79 (82.53)	0.754
LPA (min/day)	173.24 (45.15)	167.89 (37.21)	177.04 (59.55)	164.01 (43.06)	182.88 (39.56)	0.595
MVPA (min/day)	95.92 (35.62)	89.52 (29.11)	96.85 (45.30)	91.78 (34.13)	104.51 (33.42)	0.603
Dietary intake						
Energy (kcal/day)	2141 (699)	2079 (496)	2288 (1152)	2149 (514)	2054 (455)	0.767
Fat (g/day)	37.55 (6.90)	37.09 (9.20)	37.31 (8.03)	36.32 (5.93)	39.32 (4.08)	0.601
Protein (g/day)	47.14 (8.19)	49.82 (10.41)	47.85 (8.45)	47.17 (6.00)	44.21 (7.30)	0.236
Carbohydrate (g/day)	18.64 (4.91)	16.94 (4.35)	19.23 (6.84)	19.36 (4.90)	18.84 (2.97)	0.467
Energy metabolism						
BMR (kcal/day)	1508 (364)	1469 (375)	1441 (369)	1607 (415)	1511 (303)	0.562
BFox (g/min)	0.05 (0.04)	0.05 (0.04)	0.05 (0.04)	0.06 (0.05)	0.05 (0.03)	0.936
BFox (% BMR)	45.6 (30.0)	44.8 (30.0)	49.1 (34.8)	45.3 (34.6)	43.5 (22.9)	0.957
MFO (g/min)	0.29 (0.09)	0.25 (0.06)	0.28 (0.07)	0.33 (0.12)	0.29 (0.09)	0.068
MFO (mg/$kg_{leanmass}$/min)	6.78 (1.57)	5.59 (1.35)	6.77 (1.45)	7.46 (1.77)	6.77 (1.32)	0.094
Fat_{max} (% VO_2max)	43.01 (10.45)	41.51 (12.62)	44.27 (13.10)	42.88 (8.54)	43.35 (7.60)	0.896
Cardiorespiratory fitness						
VO_2max (ml/min)	2339.2 (657.2)	2163.4 (626.0)	2320.4 (649.7)	2461.8 (709.1)	2397.1 (658.3)	0.580
VO_2max (ml/kg/min)	30.49 (5.58)	28.99 (4.96)	31.64 (6.12)	31.59 (6.22)	29.74 (4.90)	0.399

Data are shown as means ± SD. Abbreviations: PAR physical activity recommendations group, HIIT high intensity interval training group, HIIT+EMS HIIT plus whole-body electromyostimulation group, LPA light physical activity, MPA moderate physical activity, VPA vigorous physical activity, MVPA moderate-vigorous physical activity, TPA total physical activity, BMR basal metabolic rate, BFox basal fat oxidation, MFO maximal fat oxidation during exercise, Fat_{max} intensity of exercise that elicits MFO, BMR basal metabolic rate VO_2max maximal oxygen uptake.

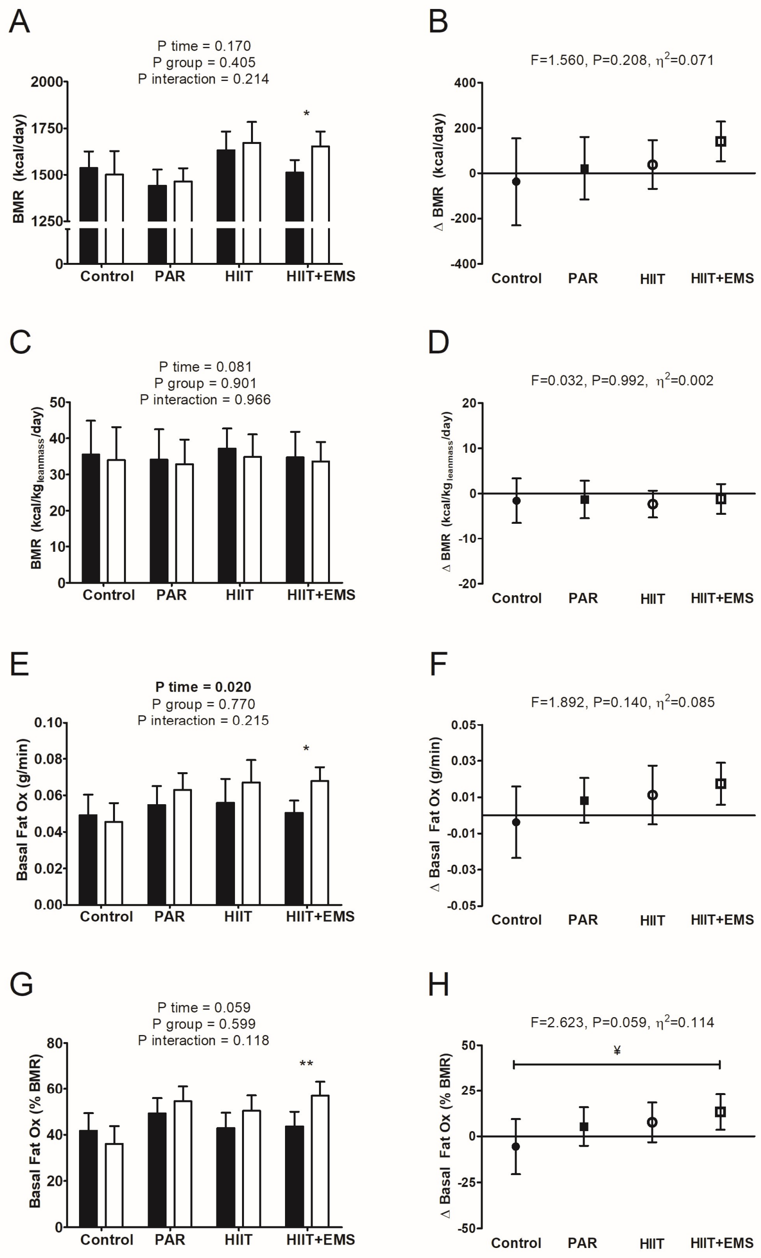

Figure 2. Basal metabolic rate (BMR) and basal fat oxidation rate (Basal Fat Ox), before and after the intervention. P value (time, group, and the interaction *time x group*) for repeated measures ANOVA (Panels A, C, E, and G). *P<0.05, **P<0.01, Student paired t test (Panels A, C, E, and G). Changes in BMR (in absolute term and relative to lean mass), and basal fat oxidation (in absolute term and expressed as %BMR), after the intervention. ¥ P<0.05, ANCOVA adjusting for baseline values, with post hoc Bonferroni-corrected t test results (Panels B, D, F, and H). ANCOVA adjusting for baseline values (Panels B, D, F, and H). Data are shown as means ± standard deviations. Abbreviations: PAR physical activity recommendations group, HIIT high intensity interval training group, HIIT+EMS HIIT plus whole-body electromyostimulation group.

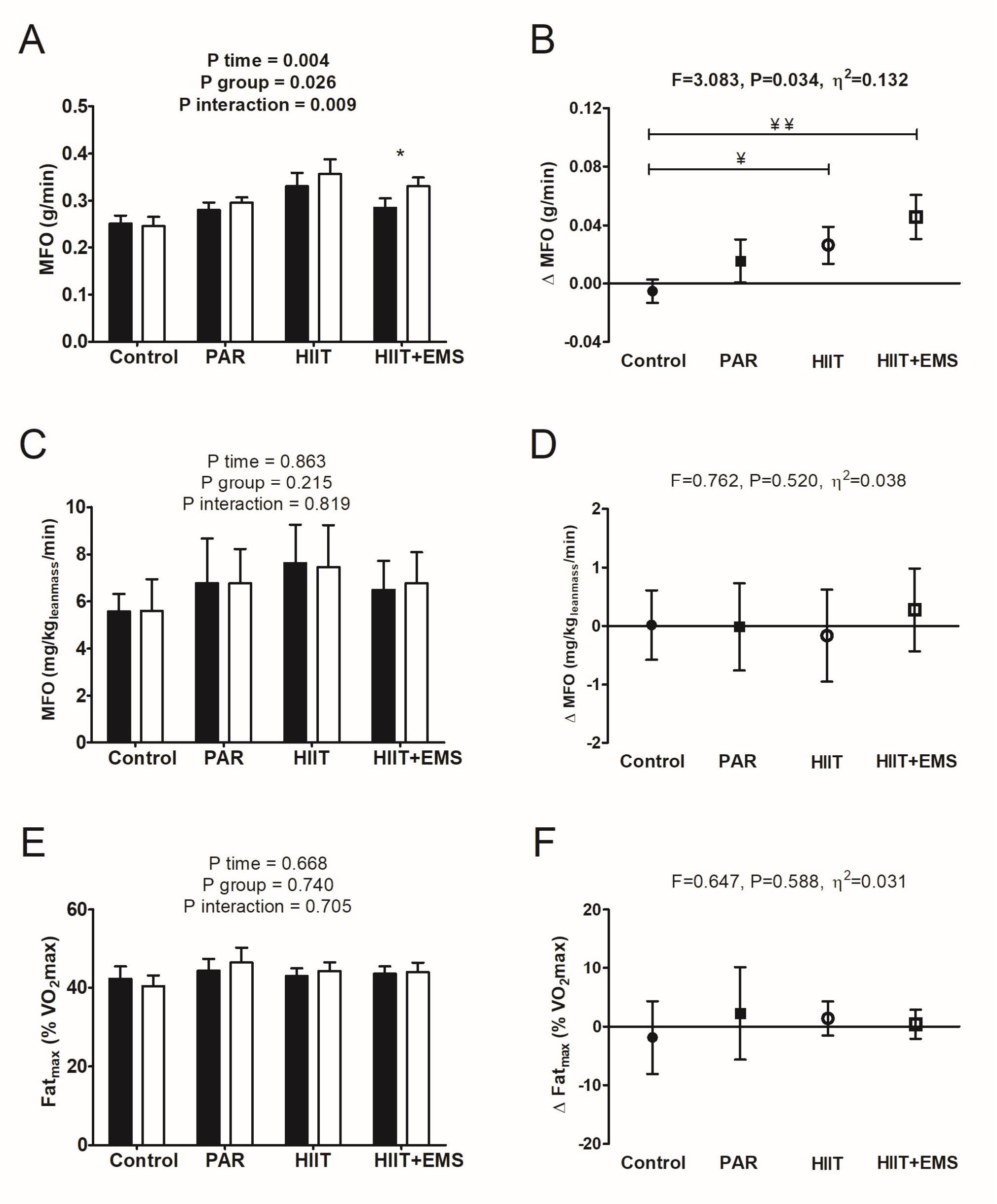

Figure 3. Maximal fat oxidation (MFO) in absolute terms and relative to the lean mass, and the intensity that of exercise that elicited the MFO (Fat_{max}), before and after the intervention. P value (time, group, and the interaction *time x group*) for repeated measures ANOVA (Panels A, C, and E). *$P<0.05$, Student paired test (Panels A, C, and E). Changes in MFO and Fat_{max} after the intervention. ¥ $P<0.05$, ¥ ¥ $P<0.01$, ANCOVA adjusting for baseline values, with post hoc Bonferroni-corrected t test results (Panels B, D, and F). The data are shown as means ± standard deviations. Abbreviations: PAR physical activity recommendations group, HIIT high intensity interval training group, HIIT+EMS HIIT plus whole-body electromyostimulation group.

Table 2. Change in body composition, blood variables, physical activity and sedentary time, dietary intake, energy metabolism and cardiorespiratory fitness, adjusted for baseline values (Model 1), for baseline values and sex (Model 2), and for baseline values and age (Model 3).

	ANCOVA P value		
	Model 1	**Model 2**	**Model 3**
Body composition			
Weight (kg)	**0.032**	**0.020**	**0.014**
BMI (kg/m^2)	**0.011**	**0.013**	**0.005**
FM (kg)	**0.014**	**0.014**	**0.025**
LM (kg)	**0.002**	**0.002**	**0.002**
Blood variables			
Plasma glucose (mg/dL)	0.638	0.691	0.671
Plasma insulin (uUI/mL)	**<0.001**	**<0.001**	**<0.001**
HOMA index	**<0.001**	**<0.001**	**<0.001**
Physical activity and sedentary time			
Sedentary time (min/day)	0.413	0.446	0.412
LPA (min/day)	0.142	0.159	0.142
MPA (min/day)	0.122	0.133	0.108
VPA (min/day)	0.457	0.494	0.483
MVPA (min/day)	0.133	0.145	0.119
TPA	0.169	0.190	0.156
Dietary intake			
Energy intake (kcal/day)	0.762	0.751	0.663
Fat (% of energy intake)	0.735	0.727	0.743
Protein (% of energy intake)	0.867	0.867	0.741
Carbohydrate (% of energy intake)	0.924	0.938	0.893
Energy metabolism			
BMR (kcal/day)	0.208	0.227	0.203
BFox (g/min)	0.140	0.153	0.106
BFox (% BMR)	0.059	0.081	0.074
MFO (g/min)	**0.034**	**0.031**	**0.039**
MFO (mg/kg$_{leanmass}$/min)	0.520	0.512	0.584
Fat$_{max}$ (% of VO_2max)	0.588	0.597	0.592
Cardiorespiratory fitness			
VO_2max (ml/min)	**0.004**	**0.001**	**0.007**
VO_2max (ml/kg/min)	**0.003**	**0.004**	**0.006**

Abbreviations: PAR physical activity recommendations group, HIIT high intensity interval training group, HIIT+EMS HIIT plus whole-body electromyostimulation group, LPA light physical activity, MPA moderate physical activity, VPA vigorous physical activity, MVPA moderate-vigorous physical activity, TPA total physical activity, BMR basal metabolic rate, BFox basal fat oxidation, MFO maximal fat oxidation during exercise, Fat$_{max}$ intensity of exercise that elicits MFO, VO_2max maximal oxygen uptake.

Table 3. Relationship of changes in body composition, blood variables, physical activity and sedentary time, dietary intake, and cardiorespiratory fitness, with the change in basal metabolic rate.

	Model 1			Model 2			Model 3		
	β	R^2	P value	β	R^2	P value	β	R^2	P value
Changes in body composition									
Weight (kg)	12.599	0.008	0.528	13.005	0.008	0.535	15.774	0.023	0.781
Body mass index (kg/m²)	25.588	0.004	0.646	25.381	0.004	0.657	36.416	0.019	0.525
Fat mass (kg)	2.986	0.004	0.659	2.749	0.005	0.694	1.416	0.020	0.614
Lean mass (kg)	-1.681	0.001	0.817	-1.485	0.002	0.841	0.734	0.019	0.924
Changes in blood variables									
Plasma glucose (mg/dL)	2.200	0.005	0.632	2.031	0.005	0.432	2.079	0.010	0.654
Plasma insulin (uUI/mL)	-7.292	0.007	0.569	-7.489	0.009	0.563	-9.369	0.016	0.479
HOMA index	-31.570	0.010	0.488	-33.253	0.012	0.472	-38.708	0.020	0.409
Changes in physical activity and sedentary time									
Sedentary time (min/day)	0.325	0.014	0.424	0.315	0.014	0.452	0.320	0.019	0.436
LPA (min/day)	-0.265	0.002	0.764	-0.245	0.003	0.785	-0.306	0.008	0.732
MPA (min/day)	-0.441	0.011	0.477	-0.432	0.012	0.491	-0.438	0.016	0.484
VPA (min/day)	-5.864	0.004	0.684	-6.157	0.006	0.673	-5.546	0.008	0.703
MVPA (min/day)	-0.432	0.011	0.476	-0.424	0.012	0.757	-0.428	0.016	0.484
TPA	-1.684	0.008	0.552	-1.630	0.009	0.570	-1.707	0.013	0.550
Changes in dietary intake									
Energy intake (kcal/day)	-0.076	0.074	0.051	-0.077	0.076	0.051	-0.073	0.078	0.065
Fat (% of energy intake)	5.560	0.038	0.170	5.553	0.038	0.175	5.151	0.042	0.218
Protein (% of energy intake)	-5.663	0.041	0.154	-5.714	0.041	0.158	-5.266	0.045	0.200
Carbohydrate (% of energy intake)	2.765	0.058	0.129	2.836	0.068	0.115	2.715	0.069	0.098
Changes in cardiorespiratory fitness									
VO_2max (ml/min)	-0.046	0.004	0.649	-0.045	0.005	0.657	-0.030	0.013	0.771
VO_2max (ml/kg/min)	-3.159	0.004	0.672	-3.158	0.004	0.676	-2.415	0.013	0.750

β = unstandardised regression coefficient, R^2 and P are from simple and multiple linear regression analysis: Model 1, simple regression analysis; Model 2, including sex in the regression model; Model 3, including age in the regression model. Abbreviations: LPA light physical activity, MPA moderate physical activity, VPA vigorous physical activity, MVPA moderate-vigorous physical activity, TPA total physical activity, VO_2max maximal oxygen uptake.

Table 4. Relationship of the changes in body composition, blood variables, physical activity and sedentary time, dietary intake, and cardiorespiratory fitness, with the change in basal fat oxidation.

	Model 1			Model 2			Model 3		
	β	R^2	P value	β	R^2	P value	β	R^2	P value
Changes in body composition									
Weight (kg)	-0.003	0.027	0.237	-0.003	0.027	0.251	-0.003	0.032	0.216
Body mass index (kg/m^2)	-0.007	0.022	0.287	-0.007	0.022	0.305	-0.008	0.027	0.256
Fat mass (kg)	**-0.002**	**0.163**	**0.003**	**-0.002**	**0.164**	**0.004**	**-0.002**	**0.164**	**0.004**
Lean mass (kg)	**0.002**	**0.139**	**0.007**	**0.002**	**0.140**	**0.008**	**0.002**	**0.143**	**0.008**
Changes in blood variables									
Plasma glucose (mg/dL)	-0.001	0.022	0.304	-0.001	0.022	0.306	-0.001	0.027	0.321
Plasma insulin (uUI/mL)	-0.001	0.005	0.632	-0.001	0.005	0.637	-0.001	0.009	0.721
HOMA index	-0.005	0.016	0.380	-0.005	0.016	0.386	-0.004	0.019	0.442
Changes in physical activity and sedentary time									
Sedentary time (min/day)	0.001	0.007	0.353	0.001	0.009	0.799	0.001	0.013	0.593
LPA (min/day)	0.211	0.044	0.151	0.210	0.044	0.158	0.220	0.055	0.137
MPA (min/day)	0.267	0.072	0.066	0.267	0.072	0.070	0.267	0.078	0.069
VPA (min/day)	0.148	0.022	0.315	0.151	0.023	0.312	0.145	0.028	0.331
MVPA (min/day)	0.268	0.072	0.066	0.267	0.072	0.070	0.267	0.078	0.069
TPA	0.292	0.085	0.064	0.292	0.085	0.067	0.294	0.093	0.054
Changes in dietary intake									
Energy intake (kcal/day)	0.038	0.001	0.788	0.041	0.003	0.773	0.033	0.002	0.231
Fat (% of energy intake)	0.260	0.067	0.066	0.259	0.067	0.069	0.260	0.067	0.075
Protein (% of energy intake)	0.131	0.017	0.359	0.136	0.018	0.351	0.152	0.025	0.304
Carbohydrate (% of ene intake)	-0.058	0.007	0.551	-0.092	0.008	0.534	-0.089	0.011	0.539
Changes in cardiorespiratory fitness									
VO_2max (ml/min)	0.157	0.025	0.262	0.155	0.025	0.274	0.157	0.025	0.279
VO_2max (ml/kg/min)	0.001	0.026	0.251	0.001	0.027	0.256	0.001	0.026	0.266

β = unstandardised regression coefficient, R^2 and P are from a simple and multiple linear regression analysis: Model 1, simple regression analysis; Model 2, including sex in the regression model; Model 3, including age in the regression model. Abbreviations: LPA light physical activity, MPA moderate physical activity, VPA vigorous physical activity, MVPA moderate-vigorous physical activity, TPA total physical activity, VO_2max maximal oxygen uptake.

Table 5. Relationship of the changes in body composition, blood variables, physical activity and sedentary time, dietary intake, and cardiorespiratory fitness, with the change in maximal fat oxidation.

	Model 1			Model 2			Model 3		
	β	R^2	P value	β	R^2	P value	β	R^2	P value
Changes in body composition									
Weight (kg)	0.005	0.019	0.321	0.004	0.028	0.447	0.004	0.073	0.467
Body mass index (kg/m^2)	0.018	0.030	0.215	0.016	0.039	0.282	0.014	0.079	0.359
Fat mass (kg)	-0.003	0.045	0.141	-0.003	0.073	0.091	-0.002	0.094	0.309
Lean mass (kg)	0.004	0.075	0.054	**0.004**	**0.095**	**0.041**	0.003	0.110	0.171
Changes in blood variables									
Plasma glucose (mg/dL)	0.001	0.020	0.325	0.001	0.025	0.397	0.001	0.075	0.259
Plasma insulin (uUI/mL)	0.000	0.000	0.960	0.000	0.010	0.996	0.002	0.055	0.636
HOMA index	0.006	0.006	0.593	0.005	0.014	0.658	0.012	0.069	0.336
Changes in physical activity and sedentary time									
Sedentary time (min/day)	-0.124	0.015	0.400	-0.163	0.051	0.278	-0.113	0.075	0.433
LPA (min/day)	0.040	0.002	0.789	0.056	0.028	0.709	0.058	0.066	0.690
MPA (min/day)	0.192	0.037	0.192	0.202	0.066	0.170	0.187	0.097	0.195
VPA (min/day)	-0.020	0.000	0.893	-0.025	0.026	0.865	-0.037	0.064	0.797
MVPA (min/day)	0.187	0.035	0.204	0.196	0.064	0.182	0.181	0.095	0.209
TPA	0.114	0.013	0.440	0.130	0.042	0.285	0.114	0.075	0.431
Changes in dietary intake									
Energy intake (kcal/day)	0.079	0.006	0.577	0.070	0.014	0.626	0.049	0.055	0.725
Fat (% of energy intake)	-0.042	0.002	0.771	-0.041	0.015	0.773	-0.005	0.040	0.973
Protein (% of energy intake)	0.035	0.001	0.806	0.052	0.016	0.720	-0.016	0.040	0.914
Carbohydrate (% of energy intake)	0.181	0.033	0.205	0.164	0.039	0.263	0.201	0.080	0.155
Changes in cardiorespiratory fitness									
VO_2max (ml/min)	**0.470**	**0.221**	**<0.001**	**0.481**	**0.246**	**<0.001**	**0.406**	**0.245**	**0.001**
VO_2max (ml/kg/min)	**0.006**	**0.197**	**0.001**	**0.006**	**0.211**	**0.001**	**0.006**	**0.236**	**0.001**

β = unstandardised regression coefficient, R^2 and P are from a simple and multiple linear regression analysis: Model 1, simple regression analysis; Model 2, including sex in the regression model; Model 3, including age in the regression model. Abbreviations: LPA light physical activity, MPA moderate physical activity, VPA vigorous physical activity, MVPA moderate-vigorous physical activity, TPA total physical activity, VO_2max maximal oxygen uptake.

DISCUSSION

The main findings of this study are that a 12 week-long, HIIT+EMS program can lead to significant improvements in BMR, BFox and MFO, in sedentary middle-aged adults. No significant improvements were seen in these areas when following a structured PAR program or a HIIT program without WB-EMS. It should be noted that the changes in BMR and MFO observed for the HIIT+EMS group were dependent on changes in LM, whereas changes in BFox were independent of this variable. These findings have important clinical implications; they suggest that a well-designed, HIIT-EMS program could help combat the appearance of chronic metabolic diseases characterized by metabolic inflexibility in sedentary middle-aged adults.

Effects of exercise training on BMR

The influence of aerobic and resistance training on BMR has been investigated in individuals with different biological characteristics, but conflicting results have been reported. While some authors report a significant increase in BMR (3-10%) [45–53], others indicate it to remain unchanged after an aerobic exercise training program [54–59].

Only a few studies have investigated their combined effects (i.e., concurrent training), with a significant increase in BMR reported after a 10-week concurrent training program in physically active men [60], but no change in BMR after a 20-week concurrent training program in sedentary middle-aged women [61] (this latter result partially agrees with the present findings).

Earlier studies investigating the effect of HIIT on BMR in healthy adults aged 18-50 years suggested significant improvements (~4%) to be made [54,62]. Although in the present study no significant difference was seen in BMR between the HIIT and the control groups after the intervention, a non-significant increase of about 3% was seen (the same magnitude as reported by the above-mentioned studies [60,61]).

There is little literature on the influence of WB-EMS on BMR. To our knowledge, just one study exists, examining the effects of a 14 week WB-EMS program on BMR in moderately-trained post-menopausal women [20]. The authors of that study reported significant improvements in body composition and muscular strength, but no significant change in BMR [20]. These results may not entirely agree with those of the present work, in which an almost significant increase in BMR was seen in the HIIT+EMS group.

When BMR was expressed relative to LM, no exercise-related changes were observed in any intervention group. The increase LM seen in response to exercise might therefore explain the increase in BMR, which supports the idea that LM is an important determinant of BMR (after all, it represents highly metabolically active tissue) [63,64]. Simple regression was performed to see if changes in LM were related to changes in BMR, and a significant positive association was obtained after adjusting for sex (P=0.041). The change in LM explained ~10% of the change in BMR, which agrees with the results of Schubert et al. [62], although Blundell et al. [63] reported the change in LM to explain ~25% of the change in BMR.

Others factors have been described that might explain the increase in BMR after an exercise training program, including (i) the upregulation of growth hormone, thyroid hormone, and catecholamines, (ii) an increase in substrate flux activity and enzymatic reactions, and (iii) increased protein synthesis [46,49,60]. Further investigations are needed to determine the signalling molecules and physiological pathways that induce the changes in BMR after following an exercise program for several weeks.

Effects of exercise training on BFox

Although the influence of different exercise training modalities on BMR has been examined in different cohorts, their effects on basal substrate metabolism have received considerably less attention. Some studies have suggested there to be a significant increase in BFox after following an aerobic exercise training program [65–67], whereas others report no effect at all [49,51,68–70]. Some earlier studies have reported no significant change to occur in BFox in response to a HIIT program in individuals of different BMI [62,71,72]; this agrees with the present results.

To our knowledge, this is the first study to show BFox to increase in response to a 12-week HIIT-EMS program. These improvements might be explained by: (i) the optimization of mitochondrial function and activity, promoting fatty-acid availability and metabolism [5], (ii) an increase in the amount and sensitivity of hormone-sensitive lipase, which improves the mobilisation of plasma fatty-acids [73], and (iii) an improvement in insulin sensitivity [5,8].

The present findings indicate changes in LM to be positively associated with changes in BFox, whereas changes in FM are negatively associated with changes in BFox. Since changes in LM and FM might be modulated by the above-mentioned mechanisms, it seems plausible that these changes may explain the observed changes in BFox. However, no improvement in BFox was seen for the PAR and HIIT groups, for reasons still unknown. It might be that WB-EMS induces a larger number of muscle contractions than possible in these other exercise training programs. The exercise-derived molecules associated with skeletal muscle contraction (such as different myokines) might act as energy metabolism regulators [73]. This needs to be examined in future studies.

Effects of exercise training on MFO

Many studies have investigated which biological and physiological factors determine MFO, with LM, VO_2max, physical activity levels and sex proposed to explain around a third of MFO [74-76]. Several variables closely related to exercise training therefore seem to strongly influence MFO. However, longitudinal studies have returned conflicting results indicating no change in MFO after either 4 weeks of continual low-intensity aerobic training or 4 weeks of interval training in middle-aged adults with obesity [74], but a sustained increase in MFO in middle-aged untrained adults after 12-months of aerobic training of moderate intensity [77] and in young sedentary overweight men after 12-weeks of aerobic training at moderate-vigorous intensity [76]. Although no significant differences were seen in MFO between the present PAR group (which involved a considerable dose of aerobic training) and the control group, a clinically important improvement in MFO was recorded in the PAR group of nearly the same magnitude (~7%) as reported in the above studies.

The influence of HIIT on MFO has also received some attention in recent years. Burgomaster et al. [78] observed a significant increase in MFO after a 6-week sprint interval training in young adults, while Talanian et al. [79] and Perry et al. [80] reported the same after 2 weeks of HIIT in recreationally active women, and after 6 weeks of HIIT in sedentary adults, respectively. These results agree with those obtained in the present work, in which similar changes in MFO were seen (~9%). However, a recent study by Astorino et al. [81] indicated no significant change in MFO in response to 20 sessions of HIIT in middle-aged active adults. These contradictory findings might be explained in that Astorino et al. [81] included active adults in their study (although other methodological differences may be important [82]), while sedentary middle-aged adults were recruited in the present work. A significant, positive association was seen between ΔVO_2max and ΔMFO, which is in agreement with the results of previous studies [75]. However, no significant association was noted between changes in any physiological variable related to MFO (LM, physical activity levels or dietary intake) and ΔMFO, which partially agrees with the findings of Astorino et al. [81].

To the best of our knowledge, this is the first study to report a significant increase in MFO after a 12 weeks of HIIT+EMS intervention (compared to a non-exercise control group). Although no significant differences were seen between the HIIT group and the HIIT+EMS group results, the ΔMFO in both exercise programs was clinically different (~9% and ~15%). Further studies with larger sample sizes are needed to confirm these findings. Interestingly, when MFO was expressed relative to LM, no exercise-related changes were observed in any intervention group. This might mean that the increase in LM in response to exercise explains the increase in MFO. In fact, a positive association was observed between changes in LM and ΔMFO. It has previously been reported that exercise training induces changes in both the epigenome, transcriptome and proteome, promoting better fuel storage and fuel oxidation in a wide range of physiological situations, thus improving metabolic flexibility during exercise [5]. These exercise-related improvements in metabolism have been ascribed to a larger mitochondrial enzyme content and increased mitochondrial activity [9,83]. Mitochondrial enzymes were not assessed in the current study, but based on the above-mentioned physiological adaptations, an increase in exercise-induced capillarisation, blood flow, and skeletal muscle proteins (e.g., UCP3) might be responsible for the change in MFO [76,84]. This hypothesis should be investigated in future work.

Methodological issues related to exercise-related changes in the BMR, BFox and MFO

The literature on the effects of exercise on BMR and fuel oxidation in basal conditions and during exercise contains conflicting findings. Some studies attribute these heterogeneous results to biological factors such as age, sex, health status or ethnicity, among others [62,76], or even to exercise training program-related factors such as modality, duration, volume or intensity [49,79,81]. However, considerably less attention has been paid to the methodological differences associated with the IC test, and in the selection and analysis of data for determining the BMR and fuel oxidation. For example, published reports have involved different metabolic carts (e.g., ParvoMedics TrueOne 2400 [Salt Lake City, Utah, USA] and the Oxycon Pro [Jaeger, Wurzburg, Germany]), and different stoichiometric equations have been used to estimate fuel oxidation (e.g., the Frayn equation [4] and the Jeukendrup equation [42]) to determine the effects of exercise on BMR, BFox and MFO [61,62,74,81]. Further, when determining BMR and basal fuel oxidation, the length of time over which subjects performed no physical activity and refrained from eating has differed, and even the data recording times have been different (30 min in some cases, through to long enough to achieve a steady state) [20,37,49,54,60]. Finally, different ergometer types have been used in different studies examining the effect of exercise training on MFO (e.g., walking or running on a treadmill vs. a cycle ergometer), as have different graded exercise protocols (e.g., different stage durations, different alterations in exercise intensities), the time interval for data selection and analysis (e.g., the last 60 s vs. the last 120 s of each stage), and data analysis techniques for estimating the MFO and Fat_{max} (e.g., measured-values vs. polynomial curves)[74,76,79,80,85]. All of these factors could influence the BMR, basal fuel oxidation, and MFO results obtained. When comparing different intervention studies, it is important to check whether the methodological details are different.

Limitations

The present work suffers from a number of limitations. Although a sample size calculation was performed (based on the results of a pilot study), the SD around the mean of the dependent variables was greater than expected, raising some concern that the study may have been underpowered for detecting differences in BMR, in BFox, and in MFO, between the different training programs. In addition, the study subjects were all sedentary middle-aged adults; the results cannot, therefore, be extrapolated to physically active, older, or younger individuals. Finally, since the regression analyses were conducted using data from the three different exercise training interventions (given the small sizes of the groups), it cannot be known whether the associations that exist within any one treatment group are the same as those that exist in any other.

CONCLUSIONS

In conclusion, the present results suggest that a 12 week-long, HIIT+EMS intervention improves BMR, BFox and MFO in sedentary middle-aged adults. Moreover, these improvements were clinically better than those induced by a 12 week-long HIIT without WB-EMS, and a 12-week-long PAR in middle-aged adults. It should be noted that the changes in BMR and MFO in response to the exercise interventions were dependent on the change in LM, whereas the change in BFox was independent of changes in LM. A well-designed, monitored, HIIT+EMS program might provide a good means of combating the appearance of chronic metabolic diseases characterised by metabolic inflexibility in sedentary middle-aged adults.

REFERENCES

1 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.

2 Amaro-Gahete FJ, Sanchez-Delgado G, Alcantara JMA, Martinez-Tellez B, Muñoz-Hernandez V, Merchan-Ramirez E *et al.* Congruent Validity of Resting Energy Expenditure Predictive Equations in Young Adults. *Nutrients* 2019; **11**: 1–13.

3 Melzer K. Carbohydrate and fat utilization during rest and physical activity. *E Spen Eur E J Clin Nutr Metab* 2011; **6**: e45–e52.

4 Frayn KN. Calculation of substrate oxidation rates in vivo from gaseous exchange. *J Appl Physiol* 1983; **55**: 628–34.

5 Goodpaster BH, Sparks LM. Metabolic Flexibility in Health and Disease. *Cell Metab* 2017; **25**: 1027–1036.

6 Galgani JE, Moro C, Ravussin E. Metabolic flexibility and insulin resistance. *AJP Endocrinol Metab* 2008; **295**: E1009–E1017.

7 Maunder E, Plews DJ, Kilding AE. Contextualising Maximal Fat Oxidation During Exercise: Determinants and Normative Values. *Front Physiol* 2018; **9**: 599.

8 Coen PM, Menshikova E V, Distefano G, Zheng D, Tanner CJ, Standley RA *et al.* Exercise and Weight Loss Improve Muscle Mitochondrial Respiration, Lipid Partitioning, and Insulin Sensitivity After Gastric Bypass Surgery. *Diabetes* 2015; **64**: 3737–50.

9 van Loon LJ, Jeukendrup AE, Saris WH, Wagenmakers AJ. Effect of training status on fuel selection during submaximal exercise with glucose ingestion. *J Appl Physiol* 1999; **87**: 1413–20.

10 Jong-Yeon K, Hickner RC, Dohm GL, Houmard JA. Long- and medium-chain fatty acid oxidation is increased in exercise-trained human skeletal muscle. *Metabolism* 2002; **51**: 460–4.

11 Piercy KL, Troiano RP, Ballard RM, Carlson SA, Fulton JE, Galuska DA *et al.* The physical activity guidelines for Americans. *JAMA - J Am Med Assoc* 2018; **320**: 2020–2028.

12 WHO | Global recommendations on physical activity for health. *WHO* 2015.

13 Trost SG, Owen N, Bauman AE, Sallis JF, Brown W. Correlates of adults' participation in physical activity: review and update. *Med Sci Sports Exerc* 2002; **34**: 1996–2001.

14 Troiano RP, Berrigan D, Dodd KW, Mâsse LC, Tilert T, McDowell M. Physical activity in the United States measured by accelerometer. *Med Sci Sports Exerc* 2008; **40**: 181–8.

15 Milanović Z, Sporiš G, Weston M. Effectiveness of High-Intensity Interval Training (HIT) and Continuous Endurance Training for VO2max Improvements: A Systematic Review and Meta-Analysis of Controlled Trials. *Sports*

Med 2015; **45**: 1469–81.

16 Wewege M, van den Berg R, Ward RE, Keech A. The effects of high-intensity interval training vs. moderate-intensity continuous training on body composition in overweight and obese adults: a systematic review and meta-analysis. *Obes Rev* 2017; **18**: 635–646.

17 Kemmler W, Weissenfels A, Willert S, Shojaa M, von Stengel S, Filipovic A *et al.* Efficacy and safety of low frequency Whole-Body Electromyostimulation (WB-EMS) to improve health-related outcomes in non-athletic adults. A systematic review. *Front Physiol* 2018; **9**.

18 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.

19 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.

20 Kemmler, Schliffka R, Mayhew JL, von Stengel S. Effects of whole-body electromyostimulation on resting metabolic rate, body composition, and maximum strength in postmenopausal women: the Training and ElectroStimulation Trial. *J strength Cond Res* 2010; **24**: 1880–7.

21 Kemmler, Teschler M, Weißenfels A, Bebenek M, Fröhlich M, Kohl M *et al.* Effects of Whole-Body Electromyostimulation versus High-Intensity Resistance Exercise on Body Composition and Strength: A Randomized Controlled Study. *Evidence-based Complement Altern Med* 2016; **3**: 44–55.

22 Filipovic A, Grau M, Kleinöder H, Zimmer P, Hollmann W, Bloch W. Effects of a Whole-Body Electrostimulation Program on Strength, Sprinting, Jumping, and Kicking Capacity in Elite Soccer Players. *J Sports Sci Med* 2016; **15**: 639–648.

23 Amaro-Gahete F, De-la-O A, Robles-González L, Castillo M, Gutierrez A. Impact of two whole-body electromyostimulation training modalities on body composition in recreational runners during endurance training cessation. *Rev Int Cienc Deporte* 2018; **53**: 205–218.

24 Kemmler W, Bebenek M, Engelke K, von Stengel S. Impact of whole-body electromyostimulation on body composition in elderly women at risk for sarcopenia: the Training and ElectroStimulation Trial (TEST-III). *Age (Dordr)* 2014; **36**: 395–406.

25 Kemmler W, Grimm A, Bebenek M, Kohl M, von Stengel S. Effects of Combined Whole-Body Electromyostimulation and Protein Supplementation on Local and Overall Muscle/Fat Distribution in Older

Men with Sarcopenic Obesity: The Randomized Controlled Franconia Sarcopenic Obesity (FranSO) Study. *Calcif Tissue Int* 2018; **103**: 1–12.

26 Kemmler W, Kohl M, Freiberger E, Sieber C, Von Stengel S. Effect of whole-body electromyostimulation and / or protein supplementation on obesity and cardiometabolic risk in older men with sarcopenic obesity: The randomized controlled FranSO trial. *BMC Geriatr* 2018; **18**: 1–12.

27 Kemmler, Teschler M, Weissenfels A, Bebenek M, von Stengel S, Kohl M *et al.* Whole-body electromyostimulation to fight sarcopenic obesity in community-dwelling older women at risk. Results of the randomized controlled FORMOsA-sarcopenic obesity study. *Osteoporos Int* 2016; **27**: 3261–3270.

28 Kemmler, von Stengel S. Whole-body electromyostimulation as a means to impact muscle mass and abdominal body fat in lean, sedentary, older female adults: subanalysis of the TEST-III trial. *Clin Interv Aging* 2013; **8**: 1353–64.

29 Schulz KF, Altman DG, Moher D, Jüni P, Altman D, Egger M *et al.* CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMC Med* 2010; **8**: 18.

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 Schulz, KF, Grimes D, Schulz KF, Grimes DA. Generation of allocation sequences in randomised trials: chance, not choice. *Lancet (London, England)* 2002; **359**: 515–9.

32 Slade SC, Dionne CE, Underwood M, Buchbinder R. Consensus on Exercise Reporting Template (CERT): Explanation and Elaboration Statement. *Br J Sports Med* 2016; **50**: 1428–1437.

33 Buchheit M, Laursen PB. High-intensity interval training, solutions to the programming puzzle: Part II: Anaerobic energy, neuromuscular load and practical applications. *Sport Med* 2013; **43**: 927–954.

34 Buchheit M, Laursen PB. High-intensity interval training, solutions to the programming puzzle: Part I: Cardiopulmonary emphasis. *Sport Med* 2013; **43**: 313–338.

35 Borg GA. Psychophysical bases of perceived exertion. *Med Sci Sports Exerc* 1982; **14**: 377–81.

36 Sanchez-Delgado G, Alcantara JMA, Ortiz-Alvarez L, Xu H, Martinez-Tellez B, Labayen I *et al.* Reliability of resting metabolic rate measurements in young adults: Impact of methods for data analysis. *Clin Nutr* 2018; **37**: 1618–1624.

37 Fullmer S, Benson-Davies S, Earthman CP, Frankenfield DC, Gradwell E, Lee PSP *et al.* Evidence analysis library review of best practices for performing indirect calorimetry in healthy and non-critically

ill individuals. *J Acad Nutr Diet* 2015; **115**: 1417–1446.e2.

38 Alcantara JMA, Sanchez-Delgado G, Martinez-Tellez B, Merchan-Ramirez E, Labayen I, Ruiz JR. Congruent validity and inter-day reliability of two breath by breath metabolic carts to measure resting metabolic rate in young adults. *Nutr Metab Cardiovasc Dis* 2018; **28**: 929–936.

39 Weir J. New methods for calculating metabolic rate with special reference to protein metabolism. *J Physiol* 1949; **109**: 1–9.

40 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; : 1–20.

41 Amaro-Gahete FJ, Sanchez-Delgado G, Ruiz JR. Commentary: Contextualising Maximal Fat Oxidation During Exercise: Determinants and Normative Values. *Front Physiol* 2018; **9**: 1460.

42 Jeukendrup AE, Wallis GA. Measurement of substrate oxidation during exercise by means of gas exchange measurements. *Int J Sports Med* 2005; **26 Suppl 1**: S28-37.

43 Amaro-Gahete FJ, Sanchez-Delgado G, Alcantara JMA, Martinez-Tellez B, Acosta FM, Helge JW *et al.* Impact of data analysis methods for maximal fat oxidation estimation during exercise in sedentary adults. *Eur J Sport Sci* 2019; : 1–10.

44 Balke B, Ware RW. An experimental study of physical fitness of Air Force personnel. *U S Armed Forces Med J* 1959; **10**: 675–88.

45 Aristizabal JC, Freidenreich DJ, Volk BM, Kupchak BR, Saenz C, Maresh CM *et al.* Effect of resistance training on resting metabolic rate and its estimation by a dual-energy X-ray absorptiometry metabolic map. *Eur J Clin Nutr* 2015; **69**: 831–6.

46 Ballor DL, Poehlman ET. Resting metabolic rate and coronary-heart-disease risk factors in aerobically and resistance-trained women. *Am J Clin Nutr* 1992; **56**: 968–74.

47 Berke EM, Gardner AW, Goran MI, Poehlman ET. Resting metabolic rate and the influence of the pretesting environment. *Am J Clin Nutr* 1992; **55**: 626–9.

48 Poehlman ET, Gardner AW, Ades PA, Katzman-Rooks SM, Montgomery SM, Atlas OK *et al.* Resting energy metabolism and cardiovascular disease risk in resistance-trained and aerobically trained males. *Metabolism* 1992; **41**: 1351–60.

49 Potteiger JA, Kirk EP, Jacobsen DJ, Donnelly JE. Changes in resting metabolic rate and substrate oxidation after 16 months of exercise training in overweight adults. *Int J Sport Nutr Exerc Metab* 2008; **18**: 79–95.

50 Sjödin AM, Forslund AH, Westerterp KR, Andersson AB, Forslund JM, Hambraeus LM. The influence of physical activity on BMR. *Med Sci Sports Exerc* 1996; **28**: 85–91.

51 Toth MJ, Poehlman ET. Resting metabolic rate and cardiovascular disease risk in

resistance- and aerobic-trained middle-aged women. *Int J Obes Relat Metab Disord* 1995; **19**: 691–8.

52 Tremblay A, Fontaine E, Poehlman ET, Mitchell D, Perron L, Bouchard C. The effect of exercise-training on resting metabolic rate in lean and moderately obese individuals. *Int J Obes* 1986; **10**: 511–7.

53 Whatley JE, Gillespie WJ, Honig J, Walsh MJ, Blackburn AL, Blackburn GL. Does the amount of endurance exercise in combination with weight training and a very-low-energy diet affect resting metabolic rate and body composition? *Am J Clin Nutr* 1994; **59**: 1088–92.

54 Broeder CE, Burrhus KA, Svanevik LS, Wilmore JH. The effects of either high-intensity resistance or endurance training on resting metabolic rate. *Am J Clin Nutr* 1992; **55**: 802–10.

55 Keytel LR, Lambert MI, Johnson J, Noakes TD, Lambert E V. Free living energy expenditure in post menopausal women before and after exercise training. *Int J Sport Nutr Exerc Metab* 2001; **11**: 226–37.

56 Meredith CN, Frontera WR, Fisher EC, Hughes VA, Herland JC, Edwards J *et al.* Peripheral effects of endurance training in young and old subjects. *J Appl Physiol* 1989; **66**: 2844–9.

57 Volpe SL, Huang HW, Larpadisorn K, Lesser II. Effect of chromium supplementation and exercise on body composition, resting metabolic rate and selected biochemical parameters in moderately obese women following an exercise program. *J Am Coll Nutr* 2001; **20**: 293–306.

58 Wilmore JH, Stanforth PR, Hudspeth LA, Gagnon J, Daw EW, Leon AS *et al.* Alterations in resting metabolic rate as a consequence of 20 wk of endurance training: the HERITAGE Family Study. *Am J Clin Nutr* 1998; **68**: 66–71.

59 Santa-Clara H, Szymanski L, Ordille T, Fernhall B. Effects of exercise training on resting metabolic rate in postmenopausal African American and Caucasian women. *Metabolism* 2006; **55**: 1358–64.

60 Dolezal BA, Potteiger JA. Concurrent resistance and endurance training influence basal metabolic rate in nondieting individuals. *J Appl Physiol* 1998; **85**: 695–700.

61 Byrne HK, Wilmore JH. The effects of a 20-week exercise training program on resting metabolic rate in previously sedentary, moderately obese women. *Int J Sport Nutr Exerc Metab* 2001; **11**: 15–31.

62 Schubert MM, Clarke HE, Seay RF, Spain KK. Impact of 4 weeks of interval training on resting metabolic rate, fitness, and health-related outcomes. *Appl Physiol Nutr Metab* 2017; **42**: 1073–1081.

63 Blundell JE, Finlayson G, Gibbons C, Caudwell P, Hopkins M. The biology of appetite control: Do resting metabolic rate and fat-free mass drive energy intake? *Physiol Behav* 2015; **152**: 473–8.

64 Caudwell P, Finlayson G, Gibbons C, Hopkins M, King N, Näslund E *et al.*

Resting metabolic rate is associated with hunger, self-determined meal size, and daily energy intake and may represent a marker for appetite. *Am J Clin Nutr* 2013; **97**: 7–14.

65 Poehlman ET, Gardner AW, Arciero PJ, Goran MI, Calles-Escandon J. Effects of endurance training on total fat oxidation in elderly persons. *J Appl Physiol* 1994; **76**: 2281–7.

66 Morio B, Montaurier C, Ritz P, Fellmann N, Coudert J, Beaufrère B *et al.* Time-course effects of endurance training on fat oxidation in sedentary elderly people. *Int J Obes Relat Metab Disord* 1999; **23**: 706–14.

67 Calles-Escandón J, Goran MI, O'Connell M, Nair KS, Danforth E. Exercise increases fat oxidation at rest unrelated to changes in energy balance or lipolysis. *Am J Physiol* 1996; **270**: E1009-14.

68 Buemann B, Astrup A, Christensen NJ. Three months aerobic training fails to affect 24-hour energy expenditure in weight-stable, post-obese women. *Int J Obes Relat Metab Disord* 1992; **16**: 809–16.

69 Kanaley JA, Cryer PE, Jensen MD. Fatty acid kinetic responses to exercise. Effects of obesity, body fat distribution, and energy-restricted diet. *J Clin Invest* 1993; **92**: 255–61.

70 Van Aggel-Leijssen DP, Saris WH, Hul GB, Van Baak MA. Long-term effects of low-intensity exercise training on fat metabolism in weight-reduced obese men. *Metabolism* 2002; **51**: 1003–10.

71 Martins C, Kazakova I, Ludviksen M, Mehus I, Wisloff U, Kulseng B *et al.* High-Intensity Interval Training and Isocaloric Moderate-Intensity Continuous Training Result in Similar Improvements in Body Composition and Fitness in Obese Individuals. *Int J Sport Nutr Exerc Metab* 2016; **26**: 197–204.

72 Fisher G, Brown AW, Bohan Brown MM, Alcorn A, Noles C, Winwood L *et al.* High Intensity Interval- vs Moderate Intensity-Training for Improving Cardiometabolic Health in Overweight or Obese Males: A Randomized Controlled Trial. *PLoS One* 2015; **10**: e0138853.

73 Jeukendrup AE, Saris WH, Wagenmakers AJ. Fat metabolism during exercise: a review. Part I: fatty acid mobilization and muscle metabolism. *Int J Sports Med* 1998; **19**: 231–44.

74 Venables MC, Jeukendrup AE. Endurance training and obesity: effect on substrate metabolism and insulin sensitivity. *Med Sci Sports Exerc* 2008; **40**: 495–502.

75 Venables MC, Achten J, Jeukendrup AE. Determinants of fat oxidation during exercise in healthy men and women: a cross-sectional study. *J Appl Physiol* 2005; **98**: 160–167.

76 Rosenkilde M, Reichkendler MH, Auerbach P, Bonne TC, Sjödin A, Ploug T *et al.* Changes in peak fat oxidation in response to different doses of endurance training. *Scand J Med Sci Sport* 2015; **25**: 41–52.

77 Scharhag-Rosenberger F, Meyer T, Walitzek S, Kindermann W. Effects of one

year aerobic endurance training on resting metabolic rate and exercise fat oxidation in previously untrained men and women. Metabolic endurance training adaptations. *Int J Sports Med* 2010; **31**: 498–504.

78 Burgomaster KA, Howarth KR, Phillips SM, Rakobowchuk M, Macdonald MJ, McGee SL *et al.* Similar metabolic adaptations during exercise after low volume sprint interval and traditional endurance training in humans. *J Physiol* 2008; **586**: 151–160.

79 Talanian JL, Galloway SDR, Heigenhauser GJF, Bonen A, Spriet LL. Two weeks of high-intensity aerobic interval training increases the capacity for fat oxidation during exercise in women. *J Appl Physiol* 2007; **102**: 1439–47.

80 Perry CGR, Heigenhauser GJF, Bonen A, Spriet LL. High-intensity aerobic interval training increases fat and carbohydrate metabolic capacities in human skeletal muscle. *Appl Physiol Nutr Metab* 2008; **33**: 1112–23.

81 Astorino TA, Edmunds RM, Clark A, Gallant R, King L, Ordille GM *et al.* Change in maximal fat oxidation in response to different regimes of periodized high-intensity interval training (HIIT). *Eur J Appl Physiol* 2018; **117**: 745–755.

82 Amaro-Gahete FJ, Ruiz JR. Methodological issues related to maximal fat oxidation rate during exercise : Comment on: Change in maximal fat oxidation in response to different regimes of periodized high-intensity interval training (HIIT). *Eur J Appl Physiol* 2018; **118**: 2029–2031.

83 Holloszy JO, Coyle EF. Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. *J Appl Physiol* 1984; **56**: 831–8.

84 Mogensen M, Bagger M, Pedersen PK, Fernström M, Sahlin K. Cycling efficiency in humans is related to low UCP3 content and to type I fibres but not to mitochondrial efficiency. *J Physiol* 2006; **571**: 669–81.

85 Nordby P, Rosenkilde M, Ploug T, Westh K, Feigh M, Nielsen NB *et al.* Independent effects of endurance training and weight loss on peak fat oxidation in moderately overweight men: a randomized controlled trial. *J Appl Physiol* 2015; **118**: 803–10.

Chapter 13:
Exercise training as a treatment for cardiometabolic risk in sedentary middle-aged adults: are physical activity guidelines the best way to improve cardiometabolic health? The FIT-AGEING randomized controlled trial
(Study 17)

ABSTRACT

This 12-week RCT investigates the effects of different training modalities on cardiometabolic risk in sedentary, middle-aged adults, and examines whether alterations in cardiometabolic risk are associated with changes in those health-related variables that are modifiable by exercise training.

The study subjects were 71 middle-aged adults (~54 years old; ~50% women) who were randomly assigned to one of the following treatment groups: 1) no exercise (control group), 2) PAR group, 3) HIIT group, or 4) HIIT+EMS group. A cardiometabolic risk score was calculated based on the International Diabetes Federation's clinical criteria.

A significant reduction in cardiometabolic risk was observed for all exercise training groups compared to the control group (all P<0.05), which persisted after adjusting potential confounders (all P<0.05). However, the HIIT+EMS group experienced the most significant reduction (P<0.001). A significant inverse relationship was detected between the change in lean mass and the change in cardiometabolic risk (P=0.045).

The 12-week exercise training programs - especially the HIIT+EMS program - significantly reduced cardiometabolic risk in sedentary, middle-aged adults independent of sex, age and cardiorespiratory fitness..

BACKGROUND

In recent decades, the worldwide prevalence of cardiovascular and chronic non-communicable metabolic disease has dramatically increased among young, middle-aged and elderly adults [1,2]. Metabolic syndrome, obesity and type II diabetes mellitus all strongly increase the risk of cardiovascular disease [3]. Changes in body composition (i.e., greater FM, larger amounts of VAT, and less LM) [4], hypertension [5], impaired glucose metabolism (i.e., the development of insulin resistance) [6], altered lipid metabolism (i.e., raised plasma triglycerides, total cholesterol and LDL-C, and reduced HDL-C) [7], low cardiorespiratory fitness [8], and an unhealthy lifestyle [9], all increase this risk.

The potential benefits of physical exercise on cardiometabolic health (independent of age, sex and other biological factors) have been well-documented [10]. International physical activity guidelines for health promotion establish that the adult population should complete at least 150 min per week of moderate intensity aerobic exercise, or 75 min per week of vigorous intensity aerobic exercise, combined with resistance training twice per week [11,12]. Previous studies have shown that concurrent training (i.e., the combination of endurance and resistance training) can substantially improve the cardiometabolic profile of healthy individuals, as well as those of patients with metabolic abnormalities [13,14]. However, the majority of people in developed societies do not meet current physical activity recommendations, a lack of time being the most commonly cited obstacle [15]. Novel, time-efficient training methods have, however, recently emerged.

Low-volume, HIIT requires relatively little time and seems capable of inducing improvements in cardiometabolic risk similar to - or even better than - that achieved by traditional endurance training at moderate intensity (which requires 400% more commitment in terms of time) [16,17]. However, there have been no studies comparing the effects of concurrent training and HIIT on the cardiometabolic risk profile of healthy or unhealthy individuals.

WB-EMS, which simultaneously stimulates up to 16 muscle groups (with different intensities per group) has recently arisen as an exercise training modality with the promise of being able to significantly improve the cardiometabolic health of elderly men [18]. We recently reported that a HIIT+EMS program enhanced the physical fitness and body composition of sedentary, middle-aged adults [19,20]. However, it remains unknown whether a HIIT+EMS program can improve the cardiometabolic profile in previously sedentary, middle-aged adults, and whether any hypothetical improvements would be greater than those obtained by HIIT alone, or those obtained by concurrent training based on international physical activity guidelines for health promotion [11,12].

The present work investigates the effects of these training modalities on cardiometabolic risk in previously sedentary, middle-aged adults, and examines whether alterations in cardiometabolic risk are associated with changes in health-related outcomes that are modifiable by exercise training (i.e. body composition, physical fitness, etc.).

MATERIAL & METHODS

Ethics statement and reporting philosophy

This study was performed as part of the FIT-AGEING project, a full description of which is available at clinicaltrial.gov: ID: NCT03334357 [21]. The present study protocol was approved by the Human Research Ethics Committee of the *Junta de Andalucía* [0838-N-2017], and complies with the latest revision of the Declaration of Helsinki. Written, informed consent was obtained from all potential participants prior to their inclusion in the project. The present text adheres to the CONSORT statement for improving the reporting of parallel group randomized trials (EQUATOR Network: http://www.equator-network.org/reporting-guidelines/consort) [22].

Study subjects and treatment groups

A total of 89 individuals (~50% women) aged 45-65 years were recruited (via local media, social networks and posters) to this 12-week, randomized, parallel group, controlled trial. The inclusion criteria were: (i) being sedentary (exercising <20 min on <3 days/week), (ii) having a stable weight over the previous 12 weeks, and (iii) having no chronic metabolic disease (e.g., diabetes mellitus type II), cardiovascular disease, cancer, or any problem that might be aggravated by exercise training.

Exercise training

The study was organized in two waves (September-December 2016 and September-December 2017) due to reasons of feasibility and practicality, and to avoid any potential seasonal bias. Using simple randomization software [23] the subjects were assigned to one of the following treatment groups: 1) no exercise (control group), 2) PAR group, 3) HIIT group, or 4) HIIT+EMS group. The team interpreting the results was blinded to the randomization process. To improve the replicability and transparency of the methodology followed, these exercise programs follow the norms of the Consensus on Exercise Reporting Template (CERT).

Individuals in the non-exercise control group were asked to maintain their physical activity levels and dietary habits over the 12-week study period. In addition, they were provided with general recommendations about a healthy lifestyle.

The PAR group subjects participated in three training sessions per week for all 12 weeks. In total, this involved 150 min/week of aerobic training at 60-65% of their HRres organized in 10 min bouts, and using either a treadmill, a cycloergometer and/or an elliptical ergometer. They also completed 60 min/week resistance training (global strength exercises including bench presses, lateral pull downs, dead lifts and squats, among others) at 40-50% of their one-maximum repetition. A recovery period of 48 h was allowed between training sessions.

The HIIT group subjects participated in two training session each week, following a long interval HIIT and a short interval HIIT protocol [24,25]. For the long interval HIIT component they exercised for 40-65 min/week at >95% of VO_2max, walking on a treadmill with a personalized slope. For the short interval HIIT component they undertook weight-bearing circuit training at level 6-9 on a perceived maximum effort scale [26]. A recovery period of 72 h was allowed to elapse between training sessions.

The HIIT+EMS subjects performed exactly the same exercise training as the HIIT group in terms of frequency, volume, intensity, type of exercise and periodization, but with additional WB-EMS. Given that the subjects had never trained with WB-EMS, a preliminary adaptational period was allowed to prevent any side effects [27]. Pulses were rectangular, bipolar and symmetrical at a frequency of 15-20 Hz in HIIT with long intervals and 35-75 Hz in HIIT with short intervals, and at an intensity of 100 mA in HIIT with long intervals and 80 mA in HIIT with short intervals. The impulse width was 200-400 μs. The duty cycle was 99% for HIIT with long intervals and 50-63% for HIIT with short intervals. All WB-EMS was provided using a Wiemspro® device (Wiemspro, Malaga, Spain), following the manufacturer's instructions.

All sessions started with a dynamic, standardized warm-up (10 min), and finished with a cooling-down protocol (active global stretching). Detailed information regarding the dose and intensity of each training intervention is available elsewhere [19,20,28]. All sessions were performed in small groups (2-6 subjects), strictly monitoring subject safety and their adherence to the required training intensity and volume. All sessions were conducted at the *Centro de Investigación Deporte y Salud (CIDS)*, University of Granada (Spain), and were monitored by exercise professionals with a degree in Sports Sciences. Training session attendance was recorded daily; repeat sessions were made available on alternative days to facilitate the recovery of any missed. A 90% minimum attendance rate was fixed for data use.

Outcomes

Anthropometry and body composition

Weight and height were measured using a SECA model 799 electronic scale and stadiometer (SECA, Hamburg, Germany). BMI was determined as *weight (kg)/height (m)*^{2}. Body composition was assessed using a Discovery Wi dual-energy X-ray absorptiometer (Hologic, Inc., Bedford, MA, USA), obtaining FM and LM following the manufacturer's recommendations.

Blood pressure

Blood pressure was determined in the right arm after a 30 min rest in a supine position, using an Omrom® HEM 705 CP automatic monitor (OMROM Health-Care Co., Kyoto, Japan), following the recommendations of the European Heart Society [29]. A minimum of three measurements were taken 1 min apart, and the mean value calculated.

Blood samples

Venous blood samples were taken from the antecubital vein and collected in EDTA tubes using the Vacutainer SST system (Becton Dickinson, Plymouth, UK). All samples were centrifuged at 4000 rpm for 7 min at 4ºC, and aliquots of plasma stored at -80ºC until analysis. Plasma glucose, total cholesterol, HDL-C, triglycerides, ALT and 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 device (Beckman Coulter, Brea, CA, USA). LDL-C was determined using the equation (*total cholesterol) – (HDL-C) – 0.45 x (triglycerides).*

Cardiometabolic risk score

The International Diabetes Federation [30] has proposed clinical criteria - WC, blood pressure, and plasma glucose, HDL-C and triglyceride concentrations - defining cardiometabolic risk. Sex-specific cardiometabolic risk scores were calculated based on these criteria. Each variable was standardized as follows: standardized value = (value – mean) / SD. The HDL-C standardized values were multiplied by -1 to represent increasing values as directly proportional to the risk score. The final score was determined as the sum of the 5 standardized scores divided by 5. The cardiometabolic risk score is a continuous variable with a mean of 0 and a SD of 1 by definition, with lower scores denoting a more favorable profile.

Fatty liver index

The fatty liver index is a validated surrogate marker of non-alcoholic fatty liver disease [31]. This was calculated from the BMI, WC, triglycerides and γ-GT using a previously validated equation [31].

QUICKI

This was calculated as the inverse of the sum of the logarithms of the plasma insulin (UI/mL) and plasma glucose (mg/dL) [32] concentrations.

HOMA

This was determined as *plasma insulin (UI/mL) x plasma glucose (nmol/L) / 22.5* [33].

Dietary intake

Dietary intake was recorded via three non-consecutive 24 h recall records, collected by a qualified nutritionist. Total energy intake and the macronutrient distribution were calculated using EvalFINUT® software, which makes use of the U.S. Department of Agriculture and the Spanish BEDCA (*Base de Datos Española de Composición de Alimentos*) databases.

Cardiorespiratory fitness

VO_2max was determined by IC using a maximum graded treadmill test following the modified Balke protocol [34] (explained in detail elsewhere) [19]. VO_2max was deemed reached when: (i) the RER was >1.1, (ii) a plateau in VO_2 (change of <100 ml/min in the last 3 consecutive 10 s stages) had been reached, and (iii) a heart rate of within 10 bpm of the age-predicted maximum was observed[35]. When these criteria were not met, peak oxygen uptake during the test was recorded [35].

Statistical analysis

Explanations of the statistical power requirements for the present work are available elsewhere [19–21,28]. Briefly, it was assumed that 25% of subjects would drop-out over the 12-week study period. Based on a pilot study, statistical power was fixed at 85% for detecting post-intervention cardiometabolic risk improvements of 10-15% (type 1 error = 0.05) [4]. A total of 20 subjects per group were necessary to meet these criteria.

Data are expressed as means (SD) unless otherwise stated. Data normality was confirmed using the Shapiro-Wilk test, visual histograms and Q-Q plots. Between-group baseline differences were examined by one-way ANOVA. Given that the aim of the study was to examine the efficacy of the exercise interventions with respect to the stated goals, per-protocol analysis was performed taking into account all subjects with a >90% attendance record for the exercise sessions. A sensitivity analysis (i.e., intention to treat analysis) was also performed using BOCF imputation for missing data. Repeated-measures ANOVA was performed to examine the changes in cardiometabolic risk score, in the QUICKI and HOMA, between groups, over time, and with respect to the interaction *group x time*.

The Student paired t test was used to study intra-group differences in dependent variables before and after the intervention. Similar analyses were conducted for anthropometric, blood pressure, Glucose metabolism, lipid metabolism and liver function-related values. ANCOVA was performed to examine the influence of the groups (fixed factor) on dependent outcomes, adjusting for baseline values (i.e., after intervention-cardiometabolic risk score minus baseline-cardiometabolic risk score). Bonferroni post hoc adjustment for multiple comparisons was used to examine differences between pairs of groups. Similar analyses were performed adjusting for age and sex as confounding variables.

Spearman correlation coefficients were also calculated to study the relationships between changes in the cardiometabolic risk score and QUICKI and HOMA values, and those in body composition, cardiorespiratory fitness and dietary variables potentially modifiable by exercise.

Calculations were preformed using the Statistical Package for the Social Sciences v.22.0 (IBM Corporation, Chicago, IL, USA). GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA) was used for plotting graphs. Significance was set at $P \leq 0.05$.

RESULTS

Figure 1 shows the flowchart for enrolment and analysis. A total of 71 participants (n=17 in the control group, n=17 in the PAR group, n=18 in the HIIT group and n=19 in the HIIT+EMS group) completed the study. Table 1 shows the descriptive characteristics of the study subjects at baseline; no significant differences between group were noted at this time.

Figure 2A shows the cardiometabolic risk score before and after the different exercise interventions. The interaction *group x time* had a significant influence on the post-intervention risk score ($P=0.002$; Figure 2A). The control group actually showed a significant increase in cardiometabolic risk score at the end of the study compared to baseline (-0.008 ± 0.088 vs. 0.128 ± 0.081; $P=0.01$; Figure 2A). Compared to the control group, however, the cardiometabolic risk score decreased in the PAR, HIIT and the HIIT+EMS groups ($P=0.026$, $P=0.041$ and $P<0.001$, respectively; Figure 2B) with no significant differences between the three groups (all $P>0.5$, Figure 2B). However, the HIIT+EMS group experienced the most significant reduction (-0.175 in the PAR group vs. -0.179 in the HIIT group vs. -0.272 in the HIIT-EMS group).

Figures 3A and 3C shows the QUICKI and HOMA indices before and after the intervention. The interaction *group x time* had a significant influence on these values ($P=0.003$ and $P=0.001$, respectively). The HIIT+EMS group showed a significant increase in the QUICKI index (0.350 ± 0.040 vs. 0.363 ± 0.039; $P<0.001$; Figure 3A), whereas the control group experienced a significant decrease (0.368 ± 0.036 vs. 0.358 ± 0.039; $P=0.04$; Figure 3A).

A significant reduction in the HOMA value was recorded for the HIIT and HIIT+EMS groups (1.654 ± 0.262 vs. 1.319 ± 0.181 for HIIT and 2.586 ± 0.586 vs. 1.980 ± 0.420 for HIIT+EMS; all $P<0.05$; Figure 3C), while a significant increase was noted for the control group (1.711 ± 0.374 vs. 2.128 ± 0.449; $P=0.05$; Figure 3C).

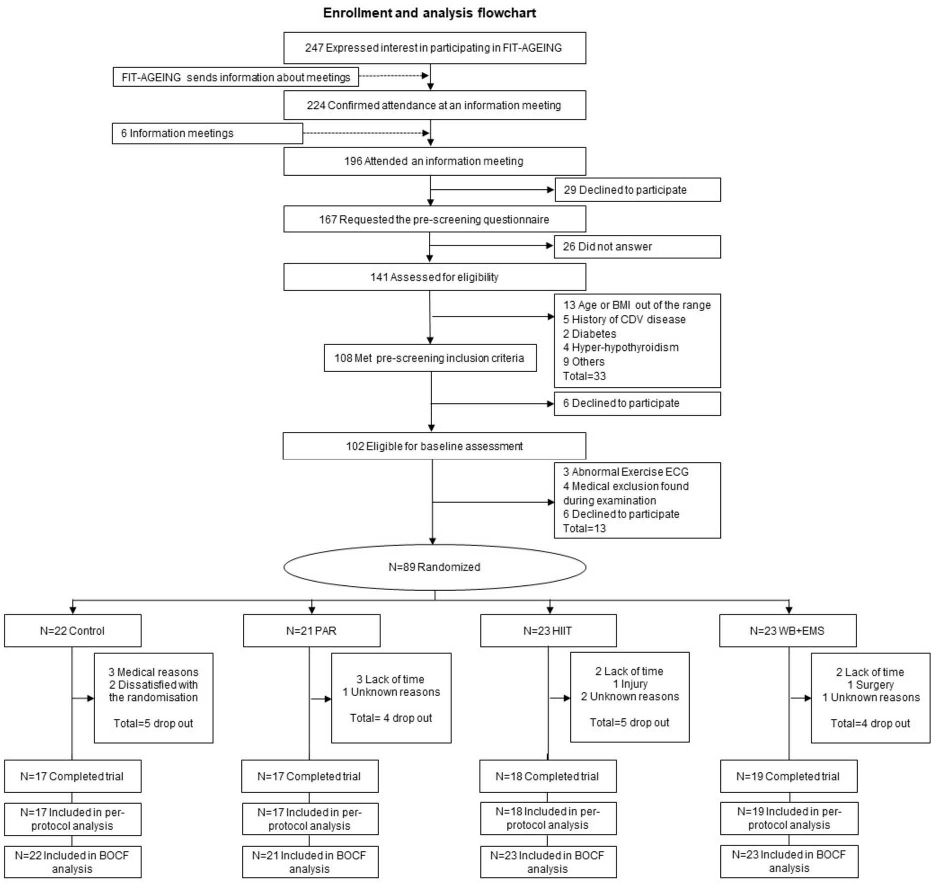

Figure 1: Enrolment and analysis flow-chart. Abbreviations: BMI - body mass index; CDV - cardiovascular disease; ECG - electrocardiogram; PAR - physical activity recommendations for adults' group; HIIT- high intensity interval training group; HIIT+EMS - HIIT plus whole-body electromyostimulation group; QUICKI - quantitative insulin sensitivity check index; BOCF - baseline observation carried forward imputation.

Table 1. Baseline descriptive characteristics of the study subjects included in the per-protocol analysis.

	All (n=71)	**Control (n=17)**	**PAR (n=17)**	**HIIT (n=18)**	**HIIT+EMS (n=19)**	**P value**
Age (years)	53.42 (4.91)	52.09 (4.05)	54.92 (4.54)	53.14 (5.59)	53.53 (5.25)	0.414
Sex (%)						
Men	34 (47.9)	7 (41.2)	8 (47.1)	9 (50.0)	10 (52.6)	
Women	37 (52.1)	10 (58.8)	9 (52.9)	9 (50.0)	9 (47.4)	
Anthropometry and body composition						
Body mass index (kg/m^2)	26.82 (3.79)	26.67 (3.71)	25.41 (2.86)	26.43 (3.15)	28.60 (4.64)	0.077
Waist circumference (cm)	95.29 (11.89)	93.35 (10.37)	90.43 (11.01)	97.53 (10.88)	99.26 (13.69)	0.107
Fat mass (kg)	30.15 (8.39)	28.64 (6.84)	26.83 (6.31)	31.42 (8.30)	33.27 (10.36)	0.097
Fat mass (%)	39.75 (8.78)	39.39 (9.30)	37.38 (8.78)	40.74 (8.56)	41.26 (8.75)	0.570
Visceral adipose tissue (g)	788.9 (391.8)	710.6 (272.4)	661.3 (262.6)	813.6 (452.2)	949.8 (477.1)	0.122
Lean mass (kg)	43.92 (11.59)	42.92 (12.06)	43.60 (10.77)	44.43 (13.52)	44.60 (10.76)	0.972
Blood pressure						
Systolic blood pressure (mm Hg)	127.09 (15.78)	127.00 (18.45)	128.88 (13.36)	126.72 (16.68)	125.88 (15.49)	0.959
Diastolic blood pressure (mm Hg)	81.12 (11.72)	82.38 (14.54)	81.75 (10.96)	80.50 (11.39)	80.0 (10.70)	0.936
Mean blood pressure (mm Hg)	104.10 (13.15)	104.69 (16.00)	105.31 (11.36)	103.61 (13.78)	102.94 (12.13)	0.957
Glucose metabolism						
Plasma glucose (mg/dL)	93.56 (11.36)	93.47 (10.82)	93.35 (11.63)	90.06 (5.56)	96.95 (14.80)	0.352
Plasma insulin (UI/mL)	8.08 (5.68)	7.26 (5.05)	7.52 (3.97)	7.09 (4.51)	10.22 (7.88)	0.296
Insulin glucose ratio	12.58 (7.56)	11.22 (6.73)	12.02 (6.23)	11.82 (7.05)	14.98 (9.57)	0.442
QUICKI	0.362 (0.036)	0.366 (0.035)	0.361 (0.032)	0.370 (0.037)	0.350 (0.040)	0.402
HOMA	1.93 (1.67)	1.73 (1.37)	1.75 (0.99)	1.59 (1.05)	2.59 (2.55)	0.255

Lipid metabolism						
Total cholesterol (mg/dL)	206.14 (32.17)	201.47 (33.98)	204.11 (17.73)	214.06 (43.34)	206.05 (28.87)	0.696
HDL-C (mg/dL)	58.71 (12.28)	61.06 (11.99)	55.18 (12.03)	57.82 (10.79)	60.58 (14.03)	0.473
LDL-C (mg/dL)	126.23 (27.07)	123.82 (28.00)	121.53 (19.74)	131.24 (35.93)	128.11 (23.77)	0.733
Triglycerides (mg/dL)	134.24 (68.16)	145.18 (81.62)	130.88 (70.00)	134.06 (61.48)	127.63 (63.27)	0.888
LDL-C/HDL-C	2.31 (0.90)	2.20 (1.01)	2.33 (0.70)	2.45 (1.12)	2.27 (0.79)	0.870
Triglycerides/HDL-C	2.57 (1.92)	2.68 (2.08)	2.67 (2.02)	2.58 (1.77)	2.37 (1.93)	0.961
Cardiometabolic risk score	-0.0002 (0.3414)	-0.0448 (0.3249)	-0.0254 (0.2822)	0.0039 (0.4164)	0.0615 (0.3460)	0.828
Liver function						
ALT (IU/L)	23.14 (12.53)	24.41 (14.51)	22.18 (10.06)	20.71 (9.74)	25.05 (15.13)	0.724
γ-GT (IU/L)	33.99 (23.26)	36.76 (27.56)	30.47 (18.12)	28.29 (17.01)	39.74 (27.64)	0.429
Fatty liver index	50.12 (26.55)	49.04 (29.04)	39.74 (23.43)	50.46 (24.87)	60.06 (26.59)	0.151
Dietary intake						
Energy (kcal/day)	2141 (699)	2079 (495)	2288 (1152)	2149 (514)	2054 (455)	0.767
Fat (g/day)	37.55 (6.90)	37.09 (9.20)	37.31 (8.03)	36.32 (5.93)	39.32 (4.08)	0.601
Carbohydrate (g/day)	47.14 (8.19)	49.82 (10.41)	47.85 (8.45)	47.17 (6.00)	44.21 (7.30)	0.236
Protein (g/day)	18.64 (4.91)	16.94 (4.35)	19.23 (6.84)	19.36 (4.90)	18.84 (2.97)	0.467
Ethanol (g/day)	10.57 (11.69)	9.43 (10.12)	9.70 (10.73)	10.64 (9.25)	12.23 (15.84)	0.894
Cardiorespiratory fitness						
VO_2max (ml/min)	2339.2 (657.2)	2163.4 (626.0)	2320.4 (649.7)	2461.8 (709.1)	2397.1 (658.3)	0.580
VO_2max_{weight} (ml/kg/min)	30.49 (5.57)	28.99 (4.96)	31.64 (6.12)	31.59 (6.22)	29.74 (4.90)	0.399

Data are shown as means (standard deviation). Abbreviations: PAR - physical activity recommendations for adults group; HIIT- high intensity interval training group; HIIT+EMS - HIIT plus whole-body electromyostimulation group; QUICKI - quantitative insulin sensitivity check index; HOMA - homeostasis model assessment index; HDL-C - high-density lipoprotein cholesterol; LDL-C - low-density lipoprotein cholesterol; ALT - Alanine transaminase; γ-GT - γ-glutamyl transferase, VO_2max - maximal oxygen uptake. P value, one-way ANOVA (to detect between-group differences at baseline).

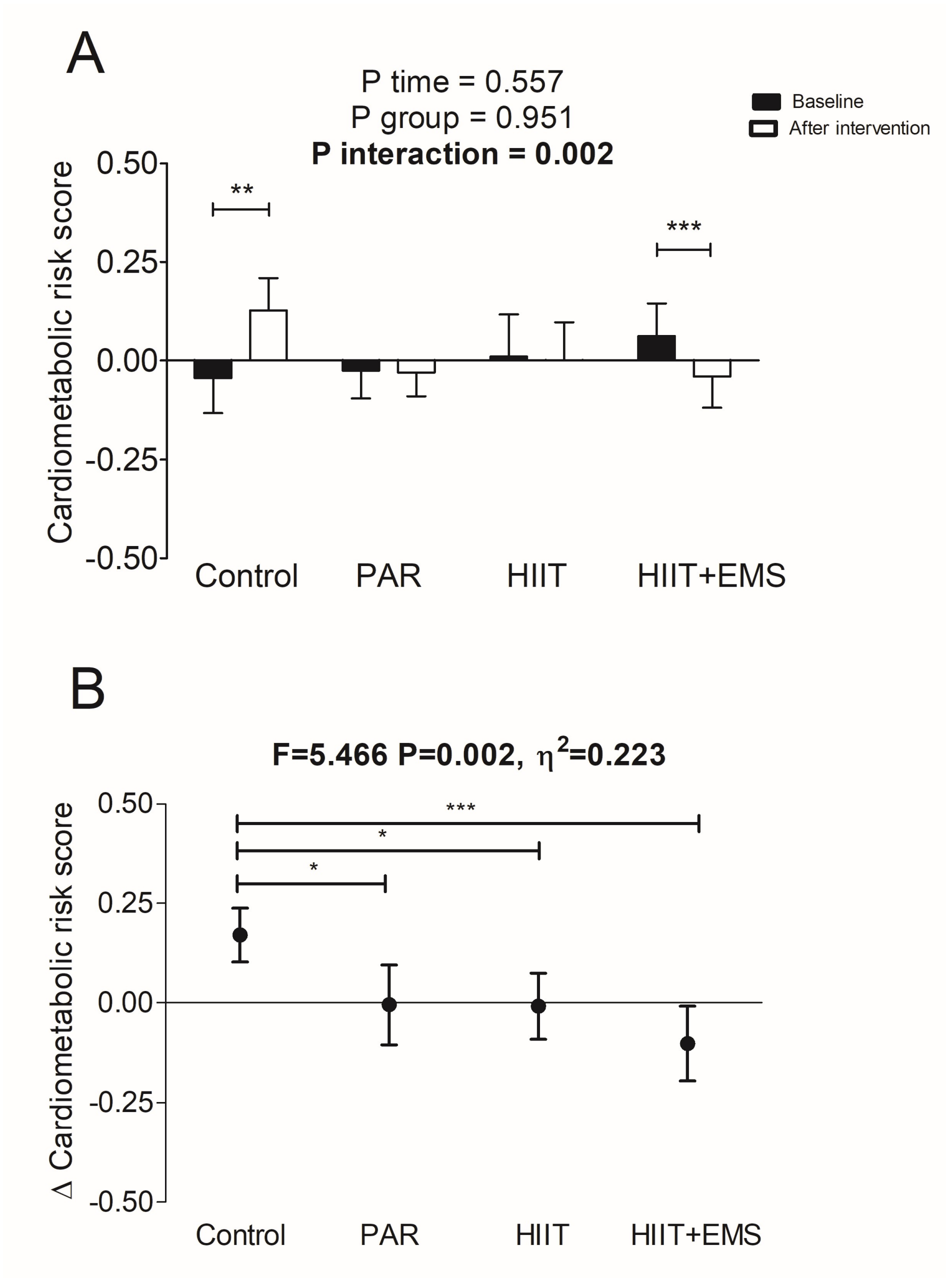

Figure 2. Cardiometabolic risk score before and after the intervention study. P value (time, group, and interaction *group x time*) for repeated measures ANOVA (Panel A). Student paired t-test to study pre-post differences (Panel A). Changes in cardiometabolic risk after the intervention study in the four groups. ANCOVA adjusting for baseline values, with post hoc Bonferroni-corrected t-test (Panel B). *P<0.05, **P<0.01, ***P<0.001. The data are shown as means (standard deviation). Abbreviations: PAR - physical activity recommendations group; HIIT - high intensity interval training group; HIIT+EMS - HIIT plus whole-body electromyostimulation group.

Compared to the control group, the QUICKI index increased significantly in all exercise intervention groups (P=0.026 for the PAR group, P=0.016 for the HIIT group, and P=0.010 for HIIT+EMS, respectively; Figure 3B), while their HOMA values fell significantly compared to the control group (P=0.002 for PAR, P=0.002 for HIIT, and P=0.001 for HIIT+EMS, respectively; Figure 3D). No significant differences were seen among the exercise intervention groups (Figure 3B and 3D).

All the above findings persisted when sex and age were included as covariates (see Table 2). Moreover, they remained consistent after performing BOCF sensitivity analysis (data not shown). Table 3 show the changes recorded in anthropometric, blood pressure, glycaemic and lipid metabolism, and liver function variables.

A significant, negative relationship was detected between the change in LM and the change in cardiometabolic risk score (P=0.045; Table 4), whereas no significant relationship was between the latter and a change in any other body composition, cardiorespiratory fitness or dietary variable (all P>0.08; Table 4). Similarly, no significant correlations were observed between changes in body composition variables, cardiorespiratory fitness or dietary variables, and changes in the QUICKI or HOMA indices (all P>0.05; Table 4).

DISCUSSION

The main finding of this work is that, compared to the control group, the HIIT+EMS subjects enjoyed the most significant improvement in cardiometabolic risk. It should be noted that, although the PAR and HIIT groups also experienced reductions in cardiometabolic risk, the improvement seen for the HIIT+EMS group was clinically greater. In addition, improvement in LM was significantly associated with a reduction in cardiometabolic risk, but no significant correlations were observed between the latter and changes in cardiorespiratory fitness or dietary variables. Taken together, these findings suggest that exercise training - especially a combination of HIIT and WB-EMS - improves cardiometabolic health in previously sedentary, middle-aged adults, independent of sex, age or cardiorespiratory fitness.

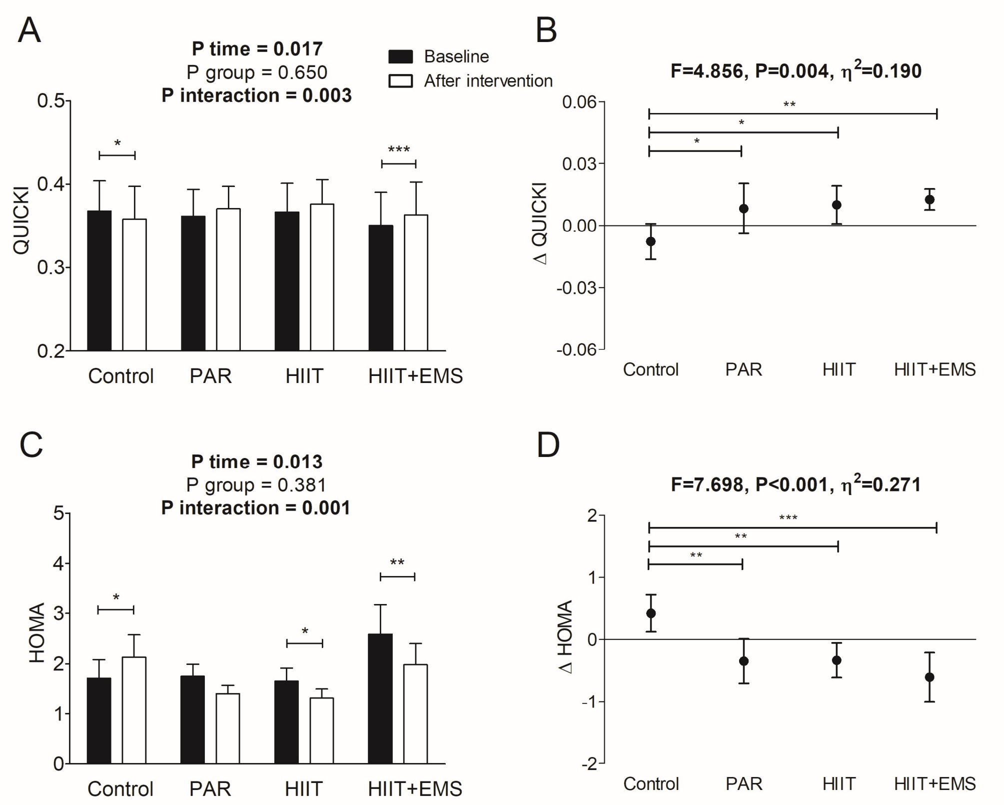

Figure 3. QUICKI (quantitative insulin sensitivity check index) and HOMA (homeostasis model assessment index) before and after the intervention study. P value (time, group, and the interaction *group x time*) for repeated measures ANOVA (Panels A and C). Student paired t-test to study pre-post differences (Panels A and C). Changes in QUICKI and HOMA after the intervention study in the four groups. P value is for ANCOVA adjusting for baseline values, with post hoc Bonferroni-corrected t-test (Panels B and D). *P<0.05, **P<0.01, ***P<0.001. Data are shown as means (standard deviation). Abbreviations: PAR - physical activity recommendations group; HIIT - high intensity interval training group; HIIT+EMS - HIIT plus whole-body electromyostimulation group.

Table 2. Changes in anthropometric variables, blood pressure, glucose and lipid metabolism, cardiometabolic risk score, and liver function adjusted for baseline values and sex (Model 1), and adjusted for baseline values and age (Model 2).

	ANCOVA P value	
	Model 1	**Model 2**
Anthropometry		
BMI (kg/m^2)	**0.013**	**0.005**
WC (cm)	**<0.001**	**<0.001**
Blood pressure		
Systolic blood pressure (mm Hg)	**<0.001**	**<0.001**
Diastolic blood pressure (mm Hg)	**<0.001**	**<0.001**
Mean blood pressure (mm Hg)	**<0.001**	**<0.001**
Glucose metabolism		
Plasma glucose (mg/dL)	0.691	0.671
Plasma insulin (UI/mL)	**<0.001**	**<0.001**
Insulin glucose ratio	**0.001**	**0.001**
QUICKI	**0.005**	**0.008**
HOMA	**<0.001**	**<0.001**
Lipid metabolism		
Total cholesterol (mg/dL)	0.116	0.079
HDL-C (mg/dL)	0.623	0.708
LDL-C (mg/dL)	**0.024**	**0.020**
Triglycerides (mg/dL)	0.097	0.264
LDL-C/HDL-C	0.368	0.421
Triglycerides/HDL-C	0.310	0.425
Cardiometabolic risk score	**0.002**	**0.003**
Liver function		
ALT (IU/L)	0.619	0.633
γ-GT (IU/L)	0.575	0.578
Fatty liver index	0.282	0.364

Abbreviations: QUICKI - quantitative insulin sensitivity check index; HOMA - homeostasis model assessment index; HDL-C - high-density lipoprotein cholesterol; LDL-C -low-density lipoprotein cholesterol; ALT - alanine transaminase, γ-GT - γ-glutamyl transferase.

Table 3. Changes in anthropometric variables, blood pressure, glycaemic and lipid metabolism, and liver function after a 12-week intervention program.

Change from Baseline at week 12	Intervention				F	P value	η^2
	Control (n=17) Mean change (SD)	PAR (n=17) Mean change (SD)	HIIT (n=18) Mean change (SD)	HIIT+EMS (n=19) Mean change (SD)			
Anthropometry							
Body mass index (kg/m^2)	-0.18 (0.34)	-0.51 (0.66)^{ab}	-0.06 (0.53)^{a}	-0.24 (0.52)^{b}	**3.993**	**0.011**	**0.160**
Waist circumference (cm)	-0.16 (2.12)^{ab}	-1.90 (3.45)	-4.53 (2.54)^{a}	-4.00 (2.37)^{b}	**7.749**	**0.011**	**0.270**
Blood pressure							
Systolic blood pressure (mm Hg)	0.38 (2.47)^{abc}	-3.50 (2.19)^{ad}	-2.06 (2.10)^{be}	-6.47 (3.34)^{cde}	**28.651**	**<0.001**	**0.593**
Diastolic blood pressure (mm Hg)	1.08 (2.63)^{abc}	-1.56 (1.90)^{ad}	-1.17 (1.92)^{be}	-4.35 (3.20)^{cde}	**17.840**	**<0.001**	**0.476**
Mean blood pressure (mm Hg)	0.73 (2.41)^{abc}	-2.53 (1.82)^{ad}	-1.61 (1.81)^{be}	-5.41 (3.14)^{cde}	**27.422**	**<0.001**	**0.582**
Glucose metabolism							
Plasma glucose (mg/dL)	-1.13 (7.75)	-2.06 (8.12)	0.56 (5.89)	-4.05 (6.28)	0.568	0.638	0.027
Plasma insulin (UI/mL)	1.93 (2.63)^{abc}	-1.37 (3.01)^{a}	-1.55 (2.66)^{b}	-1.88 (2.05)^{c}	**7.357**	**<0.001**	**0.263**
Insulin glucose ratio	3.69 (4.86)^{abc}	-1.98 (5.14)^{a}	-2.59 (4.47)^{b}	-1.86 (3.11)^{c}	**6.474**	**0.001**	**0.239**
Lipid metabolism							
Total cholesterol (mg/dL)	6.13 (38.33)	-1.00 (19.48)	-3.13 (36.54)	-15.32 (12.17)	2.230	0.093	0.097
HDL-C (mg/dL)	-0.67 (11.88)	4.71 (10.95)	5.13 (12.93)	2.21 (12.82)	0.536	0.660	0.032
LDL-C (mg/dL)	3.60 (35.82)^{a}	4.24 (21.14)^{b}	4.56 (28.84)^{b}	-18.05 (18.88)^{abc}	**3.562**	**0.019**	**0.147**
Triglycerides (mg/dL)	3.27 (57.84)^{ab}	-26.71 (60.07)^{a}	-15.44 (60.41)	-30.42 (41.09)^{b}	**3.869**	**0.013**	**0.158**
LDL-C/HDL-C	-0.01 (1.12)	-0.14 (0.58)	-0.10 (0.75)	-0.32 (0.55)	0.920	0.436	0.043
Triglycerides/HDL-C	-0.03 (1.38)	-0.76 (1.55)	-0.39 (1.38)	-0.53 (1.08)	0.929	0.432	0.043
Liver function							
ALT (IU/L)	0.47 (7.85)	-0.53 (6.77)	3.25 (7.02)	0.79 (8.72)	0.594	0.621	0.028
γ-GT (IU/L)	-2.20 (5.87)	-2.82 (7.88)	0.25 (5.16)	-0.53 (10.17)	0.680	0.568	0.032
Fatty liver index	-3.22 (7.13)	-8.85 (12.99)	-7.72 (9.04)	-10.24 (10.51)	1.167	0.330	0.054

Abbreviations: PAR - physical activity recommendations for adults group; HIIT- high intensity interval training group; HIIT+EMS - HIIT plus whole-body electromyostimulation group; QUICKI - quantitative insulin sensitivity check index; HOMA - homeostasis model assessment index; HDL-C - high-density lipoprotein cholesterol; LDL-C - low-density lipoprotein cholesterol; ALT - Alanine transaminase; γ-GT - γ-glutamyl transferase, VO_2max - maximal oxygen uptake. P value, one-way ANOVA (to detect between-group differences at baseline). P value for ANCOVA adjusting for baseline, with post hoc Bonferroni-corrected t-test (similar letters indicate significant differences).

Table 4. Spearman correlation coefficients (R_s) between changes in cardiometabolic risk, fatty liver index, Quantitative insulin sensitivity check (QUICKI) index and homeostasis model assessment (HOMA) index, and body composition, cardiorespiratory fitness (VO2max) and dietary variables (excluding control group).

	Δ Cardiometabolic risk score		Δ QUICKI index		Δ HOMA index	
	R_s	P value	R_s	P value	R_s	P value
Δ FM (%)	0.258	0.083	-0.043	0.769	0.155	0.287
Δ VAT (g)	0.227	0.130	-0.010	0.944	0.200	0.167
Δ LM (kg)	**-0.291**	**0.045**	0.071	0.629	-0.173	0.235
Δ VO_2max (ml/kg/min)	-0.108	0.461	-0.125	0.376	0.085	0.548
Δ Energy intake (kcal/day)	-0.018	0.902	-0.072	0.615	-0.027	0.852
Δ Fat (g/day)	-0.032	0.829	0.168	0.244	-0.101	0.485
Δ Carbohydrate (g/day)	0.054	0.719	0.082	0.572	-0.040	0.781
Δ Protein (g/day)	-0.206	0.164	-0.167	0.245	0.076	0.599
Δ Ethanol (g/day)	0.087	0.561	0.029	0.843	0.015	0.919

It has been reported that concurrent training can lead to cardiometabolic benefits such as reductions in WC, total cholesterol, LDL-C, triglycerides, plasma glucose and blood pressure, and an increase in HDL-C [13,36–40]. In the present study, HDL-C increased, and both total cholesterol and blood pressure decreased in the PAR group, with the changes significantly larger than those recorded for the control group. These findings agree with those of other studies involving similar exercise training interventions [13,36–38]. It should be noted that no significant differences were seen between the PAR group and the control group with respect to the change in plasma glucose concentration. However, a significant difference was seen in the change in insulin sensitivity between these two groups (higher in the PAR group). Previous studies have suggested that exercise leads to improvements in plasma glucose when the baseline levels are higher than desirable [36], but in the present work the mean baseline plasma glucose concentration of both groups was relatively normal.

It has been reported that HIIT helps reduce a number of cardiometabolic risk factors including blood pressure [41], insulin sensitivity [42] and lipogenesis [42] in individuals with different biological characteristics. A recent systematic review and meta-analysis indicated that HIIT may be a time-efficient training method in terms of improving cardiometabolic health, providing similar improvements to those achieved with continuous endurance training at moderate intensity [43]. These findings agree with those of the present study, with improvements of the same magnitude obtained in the PAR and HIIT groups.

A study that examined the effects of combining WB-EMS and whey protein supplementation on cardiometabolic risk in men aged over 70 years with sarcopenic obesity, reported a significant improvement in cardiometabolic risk after 16 weeks [18]. However, this study did not answer what the effects of an WB-EMS program without whey protein supplementation might be; or what the effects might be of a WB-EMS program on the cardiometabolic profile of sedentary men or women under 70 years of age; or whether a HIIT+EMS program might produce additional improvements in cardiometabolic risk compared with those obtained by a HIIT program without WB-EMS or with any other type of exercise training. The present work shows that a HIIT+EMS program can significantly improve the cardiometabolic profile, at least in previously sedentary, middle-aged adults compared to controls. Interestingly, although no significant differences in cardiometabolic profile were observed between the HIIT+EMS group and the HIIT or PAR groups after the corresponding interventions, a clinically relevant reduction in cardiometabolic risk was noted in the change in the HIIT+EMS group compared to the other exercise training groups, independent of sex age, or cardiorespiratory fitness. These findings suggest that a HIIT+EMS program may be the most effective training methodology for improving the cardiometabolic profile - perhaps even more so than a PAR intervention (which involves a higher exercise volume and frequency) [11,12].

The additional cardiometabolic improvements obtained by the HIIT+EMS group might be the consequence of the larger number of muscular contractions leading to a greater increase in LM [20]. Previous studies have proposed the physiological mechanisms via which an enhanced muscular mass might reduce the incidence of chronic cardiometabolic disease [13,44,45]. Skeletal muscle can be regarded as an endocrine organ since, in response to contraction, it produces myokines - molecules that play a crucial role in the modulation of obesity, metabolic syndrome, and type II diabetes mellitus [46]. It is therefore plausible that exercise-induced changes in LM can reduce cardiometabolic risk. The present results partially support this notion; a significant negative relationship was seen between the change in LM and the changes in cardiometabolic risk, but no other significant relationships were observed between changes in FM or VAT with changes in cardiometabolic risk.

It is well documented that high cardiorespiratory fitness is associated with a reduced risk of chronic cardiometabolic disease [8]. However, in addition to enhancing the former, a well-designed exercise training program should have favorable effects on glucose and lipid metabolism, and on blood pressure [10]. Certainly, some controversy surrounds the impact of changes in cardiorespiratory fitness on cardiometabolic risk, with some studies reporting an improvement to be a significant predictor of an improved glycaemic and lipid profiles [47-49], while others report no such association at all [50,51]. However, the majority of studies have been conducted in patients with cardiometabolic diseases, and have commonly involved individuals with type II diabetes mellitus. It has remained unclear whether exercise training-induced changes in cardiorespiratory fitness are related to changes in cardiometabolic risk in since sedentary, middle-aged people, who naturally have age-related increased risk of developing cardiometabolic problems [52]. The current study identified significant improvements in cardiometabolic risk for all the treatment groups, independent of changes in cardiorespiratory fitness, and even though a significant increase in cardiorespiratory fitness was seen [19]. The present lack of any association between changes in cardiorespiratory fitness and improvements in cardiometabolic profile might be explained in that exercise promotes a number of adaptive mechanisms [53]. While the enhancement of cardiorespiratory fitness in response to exercise is predominantly related to central cardiovascular adaptations, heart remodeling and an increase in stroke volume, training-associated changes in cardiometabolic profile are more related to improvements in insulin sensitivity caused by specific adaptations in adipose and skeletal muscle [10,50], an argument that the present findings support.

Limitations

The present work suffers from a number of limitations. The SD for some variables was higher than expected; the work may therefore be underpowered for detecting statistical differences between the exercise training groups with respect to some dependent outcomes. Further, insulin sensitivity/resistance was not determined by the gold standard method (i.e., the hyperinsulinemic euglycemic glucose clamp technique). However, both the QUICKI [32] and HOMA[33] methods have been validated for assessing insulin sensitivity and insulin resistance respectively.

CONCLUSIONS

In conclusion, the present results suggest that a 12-week HIIT program combined with WB-EMS can significantly reduce cardiometabolic risk in sedentary, middle-aged adults independent of sex, age and cardiorespiratory fitness. Further, exercise-induced changes in LM seem to be a powerful predictor of improvements in cardiometabolic risk after a training intervention. These results have important clinical implications: while the training intervention based on international physical activity guidelines (PAR group) improved cardiometabolic risk compared to a non-exercise control treatment, the HIIT+EMS program obtained substantially better results with less than half the training volume. Since the majority of individuals in developed countries do not meet current international physical activity recommendations, largely through a lack of time, this type of training might be particularly valuable. Further studies should be conducted to confirm these findings and to determine whether the same holds true for other populations

REFERENCES

1 Jaacks LM, Vandevijvere S, Pan A, McGowan CJ, Wallace C, Imamura F *et al.* The obesity transition: stages of the global epidemic. *Lancet Diabetes Endocrinol* 2019; **In press**.

2 Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ *et al.* Heart disease and stroke statistics--2014 update: a report from the American Heart Association. *Circulation* 2014; **129**: e28–e292.

3 Gregg EW, Cheng YJ, Cadwell BL, Imperatore G, Williams DE, Flegal KM *et al.* Secular trends in cardiovascular disease risk factors according to body mass index in US adults. *JAMA* 2005; **293**: 1868–74.

4 Shaffer ML, Chinchilli VM. Including multiple imputation in a sensitivity analysis for clinical trials with treatment failures. *Contemp Clin Trials* 2007; **28**: 130–137.

5 Perumareddi P. Prevention of Hypertension Related to Cardiovascular Disease. *Prim Care* 2019; **46**: 27–39.

6 McLaughlin T, Reaven G, Abbasi F, Lamendola C, Saad M, Waters D *et al.* Is there a simple way to identify insulin-resistant individuals at increased risk of cardiovascular disease? *Am J Cardiol* 2005; **96**: 399–404.

7 Orozco-Beltran D, Gil-Guillen VF, Redon J, Martin-Moreno JM, Pallares-Carratala V, Navarro-Perez J *et al.* Lipid profile, cardiovascular disease and mortality in a Mediterranean high-risk population: The ESCARVAL-RISK study. *PLoS One* 2017; **12**: e0186196.

8 Kaminsky LA, Arena R, Ellingsen Ø, Harber MP, Myers J, Ozemek C *et al.* Cardiorespiratory fitness and cardiovascular disease - The past, present, and future. *Prog Cardiovasc Dis* 2019.

9 Rees K, Takeda A, Martin N, Ellis L, Wijesekara D, Vepa A *et al.* Mediterranean-style diet for the primary and secondary prevention of cardiovascular disease. *Cochrane Database Syst Rev* 2019.

10 Fiuza-Luces C, Izquierdo M, Santos-Lozano A, Lucia A, Zugaza JL, Carrera-Bastos P *et al.* Exercise benefits in cardiovascular disease: beyond attenuation of traditional risk factors. *Nat Rev Cardiol* 2018; **15**: 731–743.

11 Piercy KL, Troiano RP, Ballard RM, Carlson SA, Fulton JE, Galuska DA *et al.* The physical activity guidelines for Americans. *JAMA - J Am Med Assoc* 2018; **320**: 2020–2028.

12 WHO | Global recommendations on physical activity for health. *WHO* 2015.

13 Álvarez C, Ramírez-Vélez R, Ramírez-Campillo R, Lucia A, Alonso-Martinez AM, Faúndez H *et al.* Improvements cardiometabolic risk factors in Latin American Amerindians (the Mapuche) with concurrent training. *Scand J Med Sci Sports* 2019.

14 Bennie JA, Ding D, Khan A, Stamatakis E,

Biddle SJ, Kim J. Run, lift, or both? Associations between concurrent aerobic-muscle strengthening exercise with adverse cardiometabolic biomarkers among Korean adults. *Eur J Prev Cardiol* 2018.

15 Choi J, Lee M, Lee J-K, Kang D, Choi J-Y. Correlates associated with participation in physical activity among adults: a systematic review of reviews and update. *BMC Public Health* 2017; **17**: 356.

16 Gibala MJ, Little JP, Macdonald MJ, Hawley JA. Physiological adaptations to low-volume, high-intensity interval training in health and disease. *J Physiol* 2012; **590**: 1077–84.

17 Burgomaster KA, Howarth KR, Phillips SM, Rakobowchuk M, Macdonald MJ, McGee SL *et al.* Similar metabolic adaptations during exercise after low volume sprint interval and traditional endurance training in humans. *J Physiol* 2008; **586**: 151–60.

18 Kemmler W, Kohl M, Freiberger E, Sieber C, Von Stengel S. Effect of whole-body electromyostimulation and / or protein supplementation on obesity and cardiometabolic risk in older men with sarcopenic obesity: The randomized controlled FranSO trial. *BMC Geriatr* 2018; **18**: 1–12.

19 Amaro-Gahete F, De-la-O A, Jurado-Fasoli L, Dote-Montero M, Gutiérrez A, Ruiz J *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 randomised controlled trial. *Front Physiol* 2019.

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.

21 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.

22 Schulz KF, Altman DG, Moher D, Jüni P, Altman D, Egger M *et al.* CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMC Med* 2010; **8**: 18.

23 Schulz KF, Grimes DA. Generation of allocation sequences in randomised trials: chance, not choice. *Lancet (London, England)* 2002; **359**: 515–9.

24 Buchheit M, Laursen PB. High-intensity interval training, solutions to the programming puzzle: Part II: Anaerobic energy, neuromuscular load and practical applications. *Sport Med* 2013; **43**: 927–954.

25 Buchheit M, Laursen PB. High-intensity interval training, solutions to the programming puzzle: Part I:

Cardiopulmonary emphasis. *Sport Med* 2013; **43**: 313–338.

26 Borg GA. Psychophysical bases of perceived exertion. *Med Sci Sports Exerc* 1982; **14**: 377–81.

27 Stöllberger C, Finsterer J. Side effects of whole-body electro-myo-stimulation. *Wien Med Wochenschr* 2018.

28 Amaro-Gahete F, De-la-O A, Jurado-Fasoli L, Espuch-Oliver A, de Haro T, Gutiérrez A *et al.* Exercise training increases the S-Klotho plasma levels in sedentary middle-aged adults: a randomised controlled trial. *J Sport Sci* 2019.

29 Whelton PK, Williams B. The 2018 European Society of Cardiology/European Society of Hypertension and 2017 American College of Cardiology/American Heart Association Blood Pressure Guidelines. *JAMA* 2018; **320**: 1749.

30 Carracher AM, Marathe PH, Close KL. International Diabetes Federation 2017. *J Diabetes* 2018; **10**: 353–356.

31 Bedogni G, Bellentani S, Miglioli L, Masutti F, Passalacqua M, Castiglione A *et al.* The Fatty Liver Index: a simple and accurate predictor of hepatic steatosis in the general population. *BMC Gastroenterol* 2006; **6**: 33.

32 Katz A, Nambi SS, Mather K, Baron AD, Follmann DA, Sullivan G *et al.* Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. *J Clin Endocrinol Metab* 2000; **85**: 2402–10.

33 Ascaso JF, Romero P, Real JT, Priego A, Valdecabres C, Carmena R. [Insulin resistance quantification by fasting insulin plasma values and HOMA index in a non-diabetic population]. *Med Clin (Barc)* 2001; **117**: 530–3.

34 Balke, B, Ware R. An experimental study of physical fitness of Air Force personnel. *United States Armed Forces Med Journal* 1959; **10**: 675–688.

35 Midgley AW, McNaughton LR, Polman R, Marchant D. Criteria for determination of maximal oxygen uptake: A brief critique and recommendations for future research. *Sport Med* 2007; **37**: 1019–1028.

36 Cornelissen VA, Fagard RH, Coeckelberghs E, Vanhees L. Impact of Resistance Training on Blood Pressure and Other Cardiovascular Risk Factors. *Hypertension* 2011; **58**: 950–958.

37 Greene NP, Martin SE, Crouse SF. Acute Exercise and Training Alter Blood Lipid and Lipoprotein Profiles Differently in Overweight and Obese Men and Women. *Obesity* 2012; **20**: 1618–1627.

38 Kelley GA, Kelley KS. Impact of progressive resistance training on lipids and lipoproteins in adults: a meta-analysis of randomized controlled trials. *Prev Med (Baltim)* 2009; **48**: 9–19.

39 Mann S, Beedie C, Jimenez A. Differential effects of aerobic exercise, resistance training and combined exercise modalities on cholesterol and the lipid profile: review, synthesis and

recommendations. *Sports Med* 2014; **44**: 211–21.

40 Umpierre D, Ribeiro PAB, Kramer CK, Leitão CB, Zucatti ATN, Azevedo MJ *et al.* Physical activity advice only or structured exercise training and association with HbA1c levels in type 2 diabetes: a systematic review and meta-analysis. *JAMA* 2011; **305**: 1790–9.

41 Ciolac EG, Bocchi EA, Bortolotto LA, Carvalho VO, Greve JM, Guimarães G V. Effects of high-intensity aerobic interval training vs. moderate exercise on hemodynamic, metabolic and neuro-humoral abnormalities of young normotensive women at high familial risk for hypertension. *Hypertens Res* 2010; **33**: 836–43.

42 Tjønna AE, Lee SJ, Rognmo Ø, Stølen TO, Bye A, Haram PM *et al.* Aerobic interval training versus continuous moderate exercise as a treatment for the metabolic syndrome: a pilot study. *Circulation* 2008; **118**: 346–54.

43 Batacan RB, Duncan MJ, Dalbo VJ, Tucker PS, Fenning AS. Effects of high-intensity interval training on cardiometabolic health: a systematic review and meta-analysis of intervention studies. *Br J Sports Med* 2017; **51**: 494–503.

44 Fraser BJ, Huynh QL, Schmidt MD, Dwyer T, Venn AJ, Magnussen CG. Childhood Muscular Fitness Phenotypes and Adult Metabolic Syndrome. *Med Sci Sports Exerc* 2016; **48**: 1715–22.

45 Ramírez-Vélez R, Carrillo HA, Correa-Bautista JE, Schmidt-RioValle J, González-Jiménez E, Correa-Rodríguez M *et al.* Fat-to-Muscle Ratio: A New Anthropometric Indicator as a Screening Tool for Metabolic Syndrome in Young Colombian People. *Nutrients* 2018; **10**.

46 Chung HS, Choi KM. Adipokines and Myokines: A Pivotal Role in Metabolic and Cardiovascular Disorders. *Curr Med Chem* 2018; **25**: 2401–2415.

47 Larose J, Sigal RJ, Khandwala F, Prud'homme D, Boulé NG, Kenny GP *et al.* Associations between physical fitness and $HbA_1(c)$ in type 2 diabetes mellitus. *Diabetologia* 2011; **54**: 93–102.

48 Sénéchal M, Swift DL, Johannsen NM, Blair SN, Earnest CP, Lavie CJ *et al.* Changes in body fat distribution and fitness are associated with changes in hemoglobin A1c after 9 months of exercise training: results from the HART-D study. *Diabetes Care* 2013; **36**: 2843–9.

49 Balducci S, Zanuso S, Cardelli P, Salvi L, Mazzitelli G, Bazuro A *et al.* Changes in physical fitness predict improvements in modifiable cardiovascular risk factors independently of body weight loss in subjects with type 2 diabetes participating in the Italian Diabetes and Exercise Study (IDES). *Diabetes Care* 2012; **35**: 1347–54.

50 Pandey A, Swift DL, McGuire DK, Ayers CR, Neeland IJ, Blair SN *et al.* Metabolic Effects of Exercise Training Among Fitness-Nonresponsive Patients With Type 2 Diabetes: The HART-D Study. *Diabetes Care* 2015; **38**: 1494–1501.

51 Brennan AM, Lam M, Stotz P, Hudson R, Ross R. Exercise-induced improvement in insulin sensitivity is not mediated by change in cardiorespiratory fitness. *Diabetes Care* 2014; **37**: e95-7.

52 Wilmot EG, Edwardson CL, Achana FA, Davies MJ, Gorely T, Gray LJ *et al*. Sedentary time in adults and the association with diabetes, cardiovascular disease and death: systematic review and meta-analysis. *Diabetologia* 2012; **55**: 2895–905.

53 Kokkinos P, Myers J. Exercise and physical activity: clinical outcomes and applications. *Circulation* 2010; **122**: 1637–48.

GENERAL DISCUSSION

Chapter 14:
An integrative discussion of the International Doctoral Thesis

The human race has long sought means to extend longevity and counteract the effects of ageing on physical and mental functioning. Considerable strides have been made in our biological understanding of the factors contributing to the ageing process. This knowledge is crucial for the development of therapeutic and clinical strategies to prevent, delay, or reverse age-related decline [1]. However, recent studies have suggested that chronological age is but a crude indicator of ageing. Therefore, specific ageing biomarkers have been proposed as providing a more accurate picture of the human health during the ageing process [2].

The α-Klotho gene was identified in 1997 [3] as a mutated gene in transgenic mice. It extends lifespan when overexpressed and accelerates ageing-like phenotypes when disrupted (e.g. sarcopenia, impaired cognition, atherosclerosis, endothelial dysfunction, impaired mineral metabolism, osteoporosis, growth retardation, hypokinesis and gait disturbance, skin atrophy, and emphysema) [3–6]. The α-Klotho gene encodes a single-pass transmembrane glycoprotein expressed predominantly in the distal tubule cells of the kidney, parathyroid glands, and choroid plexus of the brain. Its shed form - obtained via alternative splicing and identified in plasma, cerebrospinal fluid, and urine [7,8] - accurately indicate the α-Klotho gene expression and exerts important physiological functions [9]. Previous studies have suggested that S-Klotho are independently associated with a lower likelihood of having cardiovascular diseases [10] and that it is an independent predictor of all-cause mortality [11]. Therefore, S-Klotho has been postulated as an excellent anti-ageing biomarker [12].

However, it has not previously studied whether S-Klotho are related to well-known indicators of health during the ageing process in humans. We investigated the association between body composition (**Study 9**), physical fitness (**Study 10**), energy metabolism (**Study 11**) and cardiometabolic risk (**Study 12**) with S-Klotho in sedentary middle-aged adults. Given that we found some methodological problems regarding data collection and analysis in energy metabolism variables, we conducted some methodological studies to solve them (**Study 3, 4, 5, 6, 7 and 8**).

Moreover, previous studies have shown that physical exercise produces important benefits on human health. However, the effects of physical exercise on S-Klotho have not deeply studied. Furthermore, there is a lack of studies comparing the influence of different exercise training on the above-mentioned health-related outcomes. We systematically reviewed the literature in order to determine the role of exercise on S-Klotho protein regulation (**Study 1**) and, subsequently, we designed a randomized controlled trial (**Study 2**) aiming to determine the effects of different exercise training programs on S-Klotho (**Study 13**), body composition (**Study 14**), physical fitness (**Study 15**), energy metabolism (**Study 16**), and cardiometabolic risk (**Study 17**) in sedentary middle-aged adults.

S-KLOTHO PROTEIN AND PHYSICAL FITNESS

The body composition changes during the ageing process, decreasing LM and increasing FM [13]. Similarly, cardiorespiratory fitness and muscular strength are currently considered powerful longevity predictors and indicators of functional health [14–18]. To identify factors that play a role in the association of body composition and physical fitness with health improvements during the ageing process, is of clinical interest to understand ageing physiology. However, little is known about the association between body composition and physical fitness with the anti-ageing hormone S-Klotho. It has been shown that muscle contraction may modulate the α-Klotho gene expression [1]. A positive strong association of muscular strength [19] and functioning [20] with S-Klotho has been reported, but, the relationship between the LM and S-Klotho has not been studied in humans previously. Similarly, whether body fatness is associated with S-Klotho is unknown. Moreover, there is no studies that examine whether physical fitness is associated with S-Klotho in sedentary middle-aged adults. The results of the present International Doctoral Thesis show that LM is strongly associated with S-Klotho, and it explains the associations observed of FM with S-Klotho **(Study 9)**. Moreover, our results indicate that both cardiorespiratory fitness and muscular strength are associated with S-Klotho **(Study 10)**. Of note is, however, that these associations were highly dependent on LM. Therefore, our data support the notion that the skeletal muscle strength plays an important role in the S-Klotho metabolism, or vice versa.

S-KLOTHO PROTEIN, ENERGY METABOLISM AND CARDIOMETABOLIC HEALTH

A progressive ageing-related decline in one's metabolic and physiological functions [24], the associated dysregulation of nutrient sensitivity, mitochondrial dysfunction and cellular apoptosis eventually becoming harmful [25]. It is well-known that the ageing process is associated with a progressive decline in the BMR, meal-induced thermogenesis and physical activity [26], resulting in a reduced total energy expenditure. Moreover, several studies have examined the relationship of BFox and ageing-related diseases, and a potential role for this association has been proposed in the pathogenesis of subclinical atherosclerosis, hypertriglyceridaemia, liver steatosis and ventricular cardiac remodelling [27–29]. Paradoxically, a previous study observed no relationship between basal substrate oxidation and chronological age [24]. However, chronological age has demonstrated not to be a good indicator of ageing. It has been proposed different ageing biomarkers in the attempt of provide an accurate picture [2]. S-

Klotho exerts functions related to the physiology of energy metabolism which could modulate some cardiometabolic functions [30] (i.e. regulation of glucose uptake, enhancement of insulin sensitivity, attenuation of cellular oxidative stress, or suppression of chronic inflammation) [6,31,32]. However, the literature does not contain studies on how BMR and fuel oxidation, as well as cardiometabolic risk, may be related to S-Klotho. The findings of the present International Doctoral Thesis suggest that BFox and MFO are strongly associated with S-Klotho, while no relationship was observed between BFox and MFO with chronological age under either set of test conditions **(Study 11)**. These results have clinical implications, and support the idea that metabolic flexibility in fasting conditions and during exercise are powerful predictors of biological ageing. Finally, we also observed that S-Klotho was negatively associated with cardiometabolic risk, and positively related to insulin sensitivity/resistance independently of potential confounders such us age, cardiorespiratory fitness, physical activity levels and dietary intake **(Study 12)**. Therefore, S-Klotho would be considered a biomarker of cardiometabolic health in sedentary middle-aged individuals free of diseases.

ROLE OF EXERCISE ON S-KLOTHO PROTEIN, PHYSICAL FITNESS, ENERGY METABOLISM AND CARDIOMETABOLIC HEALTH

Increasing the amount of exercise improves physical and mental health of healthy people effectively, and prevents the development of many chronic diseases [33]. Exercise is an excellent therapeutic intervention for obesity, cardiovascular disease, type 2 diabetes, certain types of cancer, and many other chronic metabolic diseases [34]. It also has an impact on life expectancy [35], yet the physiological mechanisms that may mediate these effects are not fully understood.

ROLE OF EXERCISE ON S-KLOTHO

The role of exercise in the ageing process is well established [34,35], but the acute and chronic effects of exercise on S-Klotho have not deeply studied. Some studies observed a significant increment of S-Klotho after a single bout of exercise, concluding that healthy young well-trained individuals registered a greater improvement than the elderly untrained counterparts [36–39]. There is only one manuscript that described the chronic effect on S-Klotho after a low-to-moderate intensity aerobic training in postmenopausal women, observing a significant increase [40]. However, it remains unclear whether physical exercise can elevate S-Klotho levels, as well as which type of physical exercise induces greater improvements on S-Klotho. We showed that exercise training induced an increase in the S-Klotho in sedentary middle-aged adults and that changes in body composition were related to changes in the S-Klotho after an exercise training programme **(Study 13)**. Therefore, we suggest that the link between exercise training and the increase in S-Klotho could be mediated by a body re-composition, through a decrease of FM and an increase of LM.

ROLE OF EXERCISE ON BODY COMPOSITION

Exercise training provides important benefits on body composition and physical fitness [41]. Concurrent training induced a decrease of FM and an increment of LM and in both sedentary men [42] and women [43]. Similarly, HIIT seems to be a feasible time-efficient strategy to improve body composition [44]. Despite HIIT is currently the trendiest time-efficient exercise method, the WB-EMS [45] and has become increasingly popular during the last decade, showing that this training modality induced a generally decrease of FM and a generally increase of LM in individuals with different biological characteristics [46–55]. However, there is a lack of studies comparing the influence of different exercise training programs on body composition parameters in sedentary middle-aged adults. The results of the present International Doctoral Thesis show that both, PAR, HIIT, and HIIT+EMS, induced a decrease of FM related parameters compared to a control group, while only the HIIT with or without WB-EMS showed an increase of LM related parameters compared to the control group in sedentary middle-aged adults **(Study 14)**. Our findings suggest that a HIIT program adding or not WB-EMS, as well as a PAR program can be used as a strategy to improve body composition parameters related to high-incidence pathologies such us obesity, sarcopenia and osteoporosis, obtaining slightly better results with the application of a HIIT program adding WB-EMS.

ROLE OF EXERCISE ON PHYSICAL FITNESS

Physical exercise is currently considered an effective strategy to fight against the high prevalence of chronic cardiometabolic diseases [34], improving physical fitness, and, consequently, increasing quality of life [14–18]. It is well-known that the application of different training modalities produce important, but not similar health-related physiological adaptations [56,57]. Our findings suggest that a 12-week structured exercise intervention improves physical fitness regardless of the training programme (i.e. PAR vs. HIIT vs. HIIT+EMS) in sedentary middle-aged adults **(Study 15)**. Despite slightly greater improvements in some physical fitness variables, the changes observed in the HIIT-EMS group were not superior to the other exercise programmes.

ROLE OF EXERCISE ON ENERGY METABOLISM

The ability to increase fat oxidation under basal conditions has traditionally been regarded a powerful marker of metabolic flexibility [58]. Metabolic flexibility has been widely studied in fasting conditions, under post-prandial, and during a hyperinsulinaemic euglycaemic clamp, but its relationship with exercise and training has been much less explored [59]. MFO has been proposed key indicators of metabolic flexibility during exercise [59,60]. The development of strategies (i.e., dietary or physical exercise interventions) aimed at increasing metabolic flexibility may offer a means of combating excessive fat accumulation and obesity. Given that no previous studies have examined the influence of different exercise training programs on energy metabolism-related variables, we compared three different exercise training intentions on BMR, BFox and MFO in sedentary middle-aged adults. The results of this International Doctoral Thesis suggest that 12 week-long, HIIT+EMS program improves BMR, BFox and MFO in sedentary middle-aged adults **(Study 16)**. These findings have important clinical implications, since a well-designed, monitored, HIIT+EMS program might provide a good means of combating the appearance of chronic metabolic diseases characterised by metabolic inflexibility in sedentary middle-aged adults.

ROLE OF EXERCISE ON CARDIOMETABOLIC RISK

The worldwide prevalence of cardiovascular and chronic non-communicable metabolic disease has dramatically increased among young, middle-aged and elderly adults in the last decades [63,64]. Metabolic syndrome, obesity and type II diabetes mellitus all strongly increase the risk of cardiovascular disease [65]. Changes in body composition (i.e., greater FM, larger amounts of VAT, and less LM) [66], hypertension [67], impaired glucose metabolism [68], altered lipid metabolism [69], low cardiorespiratory fitness [70], and an unhealthy lifestyle [71], all increase this risk. The potential benefits of physical exercise on cardiometabolic health (independent of age, sex and other biological factors) have been well-documented [72]. We showed, for the first time that a 12-week HIIT program combined with WB-EMS can significantly reduce cardiometabolic risk in sedentary, middle-aged adults independent of sex, age and cardiorespiratory fitness **(Study 17)**. Further, exercise-induced changes in LM seem to be a powerful predictor of improvements in cardiometabolic risk after a training intervention. These results have important clinical implications: while the training intervention based on international physical activity guidelines also improved cardiometabolic risk compared to a non-exercise control treatment, the HIIT+EMS program obtained substantially better results with less than half the training volume. Since the majority of individuals in developed countries do not meet current international physical activity recommendations, largely through a lack of time, this type of training might be particularly valuable.

GENERAL LIMITATIONS

The findings presented in this International Doctoral Thesis should be considered cautiously since some limitations should be addressed:

- Four out of seventeen studies contained in the International Doctoral Thesis had observational designs, and thus, it is not possible to establish causal relationship.
- Six studies of the present International Doctoral Thesis included data of MFO. Given that it was calculated by a walking graded exercise protocol, we do not know whether our findings apply when MFO is calculated by a cycle ergometer graded exercise protocol.
- The results of the present International Doctoral Thesis were carried out in a cohort of sedentary middle-aged adults (45 to 65 years old) in almost cases. These data should not be extrapolated to other populations with different biological characteristics. Therefore, it is mandatory to replicate this intervention on different populations.
- A total of five studies of this International Doctoral Thesis included data of S-Klotho. However, S-Klotho might not sufficiently reflect neither tissue levels of Klotho protein nor Klotho gene expression, which cannot be obtained and analyzed in the absence of a clinical indication for biopsy.
- Several studies of the present International Doctoral Thesis included IC data. To do so, we used a metabolic cart with a relatively low inter-day reliability [73]. Thus, device error might have contributed to intra-individual non-biological variance.
- Although we conducted a sample size calculation (based on the results of a pilot study), the SD around the mean of the some dependent variables was greater than expected, raising some concern that the study may have been underpowered for detecting differences in S-Klotho, body composition, physical fitness, energy metabolism, and cardiometabolic risk, between the different training programs.

REFERENCES

1 Avin KG, Coen PM, Huang W, Stolz DB, Sowa GA, Dubé JJ *et al.* Skeletal muscle as a regulator of the longevity protein, Klotho. *Front Physiol* 2014; **5**: 189.

2 Foo H, Mather KA, Thalamuthu A, Sachdev PS. The many ages of man: diverse approaches to assessing ageing-related biological and psychological measures and their relationship to chronological age. *Curr Opin Psychiatry* 2019; **32**: 130–137.

3 Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T *et al.* Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* 1997; **390**: 45–51.

4 Iida R, Kanko S, Suga T, Morito M, Yamame A. Autophagic-lysosomal pathway functions in the masseter and tongue muscles in the klotho mouse, a mouse model for aging. *Mol Cell Biochem* 2011; **348**: 89–98.

5 Saito Y, Yamagishi T, Nakamura T, Ohyama Y, Aizawa H, Suga T *et al.* Klotho protein protects against endothelial dysfunction. *Biochem Biophys Res Commun* 1998; **248**: 324–9.

6 Kuro-o M. Klotho. *Pflugers Arch Eur J Physiol* 2010; **459**: 333–343.

7 Imura A, Iwano A, Tohyama O, Tsuji Y. Secreted Klotho protein in sera and CSF: implication for post-translational cleavage in release of Klotho protein from cell membrane. *FEBS Lett* 2004; **565**: 143–147.

8 Kim J-H, Hwang K-H, Park K-S, Kong ID, Cha S-K. Biological role of anti-aging protein Klotho. *J lifestyle Med* 2015; **5**: 1–6.

9 Kuro-o M. The Klotho proteins in health and disease. *Nat Rev Nephrol* 2019; **15**: 27–44.

10 Semba R, Cappola A, Sun K. Plasma klotho and cardiovascular disease in adults. *J Am Geriatr Soc* 2011; **59**: 1596–1601.

11 Semba RD, Cappola AR, Sun K, Bandinelli S, Dalal M, Crasto C *et al.* Plasma klotho and mortality risk in older community-dwelling adults. *Journals Gerontol - Ser A Biol Sci Med Sci* 2011; **66 A**: 794–800.

12 Chen C, Podvin S, Gillespie E, Leeman SE, Abraham CR. Insulin stimulates the cleavage and release of the extracellular domain of Klotho by ADAM10 and ADAM17. *PNAS* 2007; **104**: 19796–19801.

13 Kohara K. Sarcopenic obesity in aging population: current status and future directions for research. *Endocrine* 2014; **45**: 15–25.

14 García-Hermoso A, Cavero-Redondo I, Ramírez-Vélez R, Ruiz JR, Ortega FB, Lee DC *et al.* Muscular Strength as a Predictor of All-Cause Mortality in an Apparently Healthy Population: A Systematic Review and Meta-Analysis of Data From Approximately 2 Million Men and Women. *Arch Phys Med Rehabil* 2018.

15 Volaklis K, Halle M, Meisinger. Muscular strength as a strong predictor of mortality: A narrative review. *Eur J Intern Med* 2015; **26**: 303–310.

16 Lee, M, Artero, EG, Sui, X, Blair S, Lee D, Artero EG, Sui X, Blair SN. Mortality trends in the general population: the importance of cardiorespiratory fitness. *J Psychopharmacol* 2010; **24**: 27–35.

17 Harber M, Kaminsky L, Arena R, Blair S, Franklin B, Myers J *et al.* Impact of cardiorespiratory fitness on all-cause and disease-specific mortality: advances since 2009. *Prog Cardiovasc Dis* 2017.

18 Fitzgerald S, Barlow C, Kampert J, Morrow J, Jackson A, Blair S. Muscular fitness and all-cause mortality: prospective observations. *J Phys Act Heal* 2004; **1**: 7–18.

19 Semba RRD, Cappola ARA, Sun K, Bandinelli S, Dalal M, Crasto C *et al.* Relationship of low plasma klotho with poor grip strength in older community-dwelling adults: the InCHIANTI study. *Eur J Appl Physiol* 2012; **112**: 1215–20.

20 Crasto C, Semba R, Sun K, Cappola A, Bandinelli S, Ferrucci L. Relationship of low-circulating "anti-aging" klotho hormone with disability in activities of daily living among older community-dwelling adults. *Rejuvenation Res* 2012; **5**: 295–301.

21 Yamada Y, Ando F, Niino N, Shimokata H. Association of polymorphisms of the androgen receptor and klotho genes with bone mineral density in Japanese women. *J Mol Med* 2005; **83**: 50–57.

22 Zarrabeitia MT, Hernández JL, Valero C, Zarrabeitia AL, Ortiz F, Gonzalez-Macias J *et al.* Klotho gene polymorphism and male bone mass. *Calcif Tissue Int* 2007; **80**: 10–14.

23 Chalhoub D, Marques E, Meirelles O, Semba RD, Ferrucci L, Satterfield S *et al.* Association of Serum Klotho with Loss of Bone Mineral Density and Fracture Risk in Older Adults. *J Am Geriatr Soc* 2016; **64**: e304–e308.

24 Siervo M, Lara J, Celis-Morales C, Vacca M, Oggioni C, Battezzati A *et al.* Age-related changes in basal substrate oxidation and visceral adiposity and their association with metabolic syndrome. *Eur J Nutr* 2016; **55**: 1755–67.

25 López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. *Cell* 2013; **153**.

26 St-Onge M-P, Gallagher D. Body composition changes with aging : The cause or the result of alterations in metabolic rate and macronutrient oxidation ? *Nutrition* 2010; **26**: 152–155.

27 Montalcini T, Lamprinoudi T, Gorgone G, Ferro Y, Romeo S, Pujia A. Subclinical cardiovascular damage and fat utilization in overweight/obese individuals receiving the same dietary and pharmacological interventions. *Nutrients* 2014; **6**: 5560–71.

28 Montalcini T, Lamprinoudi T, Morrone A, Mazza E, Gazzaruso C, Romeo S *et al.* Nutrients utilization in obese individuals with and without hypertriglyceridemia. *Nutrients* 2014; **6**: 790–8.

29 Montalcini T, Gazzaruso C, Ferro Y, Migliaccio V, Rotundo S, Castagna A *et al.*

Metabolic fuel utilization and subclinical atherosclerosis in overweight/obese subjects. *Endocrine* 2013; **44**: 380–5.

30 Razzaque MS. The role of Klotho in energy metabolism. *Nat Rev Endocrinol* 2012; **8**: 579–587.

31 Kuro-o M. Molecular Mechanisms Underlying Accelerated Aging by Defects in the FGF23-Klotho System. *Int J Nephrol* 2018; **2018**: 1–6.

32 Kuro-o M. Klotho and the Aging Process. *Korean J Intern Med* 2011; **26**: 113.

33 Darren W, Crystal-Whitney N, Shannon B. Health benefits of physical activity: the evidence. *Can Med Assoc J* 2006; **174**: 801–809.

34 Pedersen B, Saltin B. Exercise as medicine - Evidence for prescribing exercise as therapy in 26 different chronic diseases. *Scand J Med Sci Sport* 2015; **25**: 1–72.

35 Gremeaux V, Gayda M, Lepers R, Sosner P, Juneau M, Nigam A. Exercise and longevity. *Maturitas* 2012; **73**: 312–317.

36 Saghiv M, Goldhammer E, Sagiv M, Ben-Sira D. Effects of aerobic exercise training on S-Klotho in young and elderly. *J J Physiol* 2015; **1**: 1–2.

37 Saghiv M, Sagiv M, Ben-Sira D. Comparison between aerobic and anaerobic training influence on s-klotho blood levels following 60 min aerobic bout. *J J Physiol* 2015; **1**: 1–4.

38 Santos-Dias A, MacKenzie B, Oliveira-Junior MC, Moyses RM, Consolim-Colombo FM, Vieira RP. Longevity protein klotho is induced by a single bout of exercise. *Br J Sports Med* 2017; **51**: 549–550.

39 Mostafidi, E, Moeen, A, Nasri, H, Ghorbani-Hagjo, A, Ardalan M. Serum Klotho levels in trained athletes. *Nephrourol Mon* 2016; **8**: 12–17.

40 Matsubara T, Miyaki A, Akazawa N, Choi Y, Ra S-G, Tanahashi K *et al.* Aerobic exercise training increases plasma Klotho levels and reduces arterial stiffness in postmenopausal women. *Am J Physiol Heart Circ Physiol* 2014; **306**: H348-55.

41 Hita-Contreras F, Martínez-Amat A, Cruz-Díaz D, Pérez-López FR. Osteosarcopenic obesity and fall prevention strategies. *Maturitas* 2015; **80**: 126–132.

42 Michell V, Samaria C, Júnior Rudy N, Danyela V, Dantas E. Effects of a concurrent physical exercise program on aerobic power and body composition in adults. *J Sports Med Phys Fitness* 2014; **54**: 441–6.

43 Ferreira FC, de Medeiros AI, Nicioli C, Nunes JED, Shiguemoto GE, Prestes J *et al.* Circuit resistance training in sedentary women: body composition and serum cytokine levels. *Appl Physiol Nutr Metab* 2010; **35**: 163–171.

44 Wewege M, van den Berg R, Ward RE, Keech A. The effects of high-intensity interval training vs. moderate-intensity continuous training on body composition in overweight and obese adults: a systematic review and meta-analysis. *Obes Rev* 2017; **18**: 635–646.

45 Kemmler W, Weissenfels A, Willert S, Shojaa M, von Stengel S, Filipovic A *et al.* Efficacy and safety of low frequency Whole-Body Electromyostimulation (WB-EMS) to improve health-related outcomes in non-athletic adults. A systematic review. *Front Physiol* 2018; **9**.

46 Amaro-Gahete F, De-la-O A, Robles-González L, Castillo M, Gutierrez A. Impact of two whole-body electromyostimulation training modalities on body composition in recreational runners during endurance training cessation. *Rev Int Cienc Deporte* 2018; **53**: 205–218.

47 Kemmler W, Grimm A, Bebenek M, Kohl M, von Stengel S. Effects of Combined Whole-Body Electromyostimulation and Protein Supplementation on Local and Overall Muscle/Fat Distribution in Older Men with Sarcopenic Obesity: The Randomized Controlled Franconia Sarcopenic Obesity (FranSO) Study. *Calcif Tissue Int* 2018; **103**: 1–12.

48 Kemmler W, Weissenfels A, Teschler M, Willert S, Bebenek M, Shojaa M *et al.* Whole-body electromyostimulation and protein supplementation favorably affect sarcopenic obesity in community-dwelling older men at risk: The randomized controlled FranSO study. *Clin Interv Aging* 2017; **12**: 1503–1513.

49 Kemmler W, Bebenek M, Engelke K, von Stengel S. Impact of whole-body electromyostimulation on body composition in elderly women at risk for sarcopenia: the Training and ElectroStimulation Trial (TEST-III). *Age (Dordr)* 2014; **36**: 395–406.

50 Kemmler, Schliffka R, Mayhew JL, von Stengel S. Effects of whole-body electromyostimulation on resting metabolic rate, body composition, and maximum strength in postmenopausal women: the Training and ElectroStimulation Trial. *J strength Cond Res* 2010; **24**: 1880–7.

51 Kemmler, Teschler M, Weissenfels A, Bebenek M, von Stengel S, Kohl M *et al.* Whole-body electromyostimulation to fight sarcopenic obesity in community-dwelling older women at risk. Results of the randomized controlled FORMOsA-sarcopenic obesity study. *Osteoporos Int* 2016; **27**: 3261–3270.

52 Kemmler, Teschler M, Weißenfels A, Bebenek M, Fröhlich M, Kohl M *et al.* Effects of Whole-Body Electromyostimulation versus High-Intensity Resistance Exercise on Body Composition and Strength: A Randomized Controlled Study. *Evidence-based Complement Altern Med* 2016; **3**: 44–55.

53 Kemmler, von Stengel S. Whole-body electromyostimulation as a means to impact muscle mass and abdominal body fat in lean, sedentary, older female adults: subanalysis of the TEST-III trial. *Clin Interv Aging* 2013; **8**: 1353–64.

54 Von Stengel S, Bebenek M, Engelke K, Kemmler W. Whole-Body

Electromyostimulation to Fight Osteopenia in Elderly Females: The Randomized Controlled Training and Electrostimulation Trial (TEST-III). *J Osteoporos* 2015; **11**: 1–7.

55 Kemmler W, Kohl M, Freiberger E, Sieber C, Von Stengel S. Effect of whole-body electromyostimulation and / or protein supplementation on obesity and cardiometabolic risk in older men with sarcopenic obesity: The randomized controlled FranSO trial. *BMC Geriatr* 2018; **18**: 1–12.

56 Huang G, Gibson CA, Tran Z V, Osness WH. Controlled endurance exercise training and VO2max changes in older adults: a meta-analysis. *Prev Cardiol* 2005; **8**: 217–25.

57 Liu C-J, Latham NK. Progressive resistance strength training for improving physical function in older adults. *Cochrane database Syst Rev* 2009; **8**: CD002759.

58 Galgani JE, Moro C, Ravussin E. Metabolic flexibility and insulin resistance. *AJP Endocrinol Metab* 2008; **295**: E1009–E1017.

59 Goodpaster BH, Sparks LM. Metabolic Flexibility in Health and Disease. *Cell Metab* 2017; **25**: 1027–1036.

60 Maunder E, Plews DJ, Kilding AE. Contextualising Maximal Fat Oxidation During Exercise: Determinants and Normative Values. *Front Physiol* 2018; **9**: 599.

61 WHO | Global recommendations on physical activity for health. *WHO* 2015.

62 Piercy KL, Troiano RP, Ballard RM, Carlson SA, Fulton JE, Galuska DA *et al.* The physical activity guidelines for Americans. *JAMA - J Am Med Assoc* 2018; **320**: 2020–2028.

63 Jaacks LM, Vandevijvere S, Pan A, McGowan CJ, Wallace C, Imamura F *et al.* The obesity transition: stages of the global epidemic. *Lancet Diabetes Endocrinol* 2019; **In press**.

64 Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ *et al.* Heart disease and stroke statistics--2014 update: a report from the American Heart Association. *Circulation* 2014; **129**: e28–e292.

65 Gregg EW, Cheng YJ, Cadwell BL, Imperatore G, Williams DE, Flegal KM *et al.* Secular trends in cardiovascular disease risk factors according to body mass index in US adults. *JAMA* 2005; **293**: 1868–74.

66 Shaffer ML, Chinchilli VM. Including multiple imputation in a sensitivity analysis for clinical trials with treatment failures. *Contemp Clin Trials* 2007; **28**: 130–137.

67 Perumareddi P. Prevention of Hypertension Related to Cardiovascular Disease. *Prim Care* 2019; **46**: 27–39.

68 McLaughlin T, Reaven G, Abbasi F, Lamendola C, Saad M, Waters D *et al.* Is there a simple way to identify insulin-resistant individuals at increased risk of cardiovascular disease? *Am J Cardiol* 2005; **96**: 399–404.

69 Orozco-Beltran D, Gil-Guillen VF, Redon

J, Martin-Moreno JM, Pallares-Carratala V, Navarro-Perez J *et al.* Lipid profile, cardiovascular disease and mortality in a Mediterranean high-risk population: The ESCARVAL-RISK study. *PLoS One* 2017; **12**: e0186196.

70 Kaminsky LA, Arena R, Ellingsen Ø, Harber MP, Myers J, Ozemek C *et al.* Cardiorespiratory fitness and cardiovascular disease - The past, present, and future. *Prog Cardiovasc Dis* 2019.

71 Rees K, Takeda A, Martin N, Ellis L, Wijesekara D, Vepa A *et al.* Mediterranean-style diet for the primary and secondary prevention of cardiovascular disease. *Cochrane Database Syst Rev* 2019.

72 Fiuza-Luces C, Izquierdo M, Santos-Lozano A, Lucia A, Zugaza JL, Carrera-Bastos P *et al.* Exercise benefits in cardiovascular disease: beyond attenuation of traditional risk factors. *Nat Rev Cardiol* 2018; **15**: 731–743.

73 Alcantara JMA, Sanchez-Delgado G, Martinez-Tellez B, Merchan-Ramirez E, Labayen I, Ruiz JR. Congruent validity and inter-day reliability of two breath by breath metabolic carts to measure resting metabolic rate in young adults. *Nutr Metab Cardiovasc Dis* 2018; **28**: 929–936.

CONCLUSIONS

GENERAL CONCLUSION

The results of the present International Doctoral Thesis show that S-Klotho is strongly related to physical fitness, energy metabolism and cardiometabolic health in sedentary middle-aged adults. Moreover, a 12-weeks structured exercise training program significantly increases S-Klotho, physical fitness, energy metabolism and cardiometabolic health in sedentary middle-aged adults.

SPECIFIC CONCLUSIONS

SECTION 1: S-Klotho protein and physical fitness

- Higher LM are associated with higher levels of S-Klotho, which explains the associations of BMI, BMD, and physical fitness with S-Klotho in sedentary middle-aged adults

SECTION 2: S-Klotho protein, energy metabolism and cardiometabolic health

- Higher BFox and MFO are associated with higher levels of S-Klotho in sedentary middle-aged adults.
- Higher levels of S-Klotho are associated with better cardiometabolic healhty, and positively related to insulin sensitivity/resistance in healthy sedentary middle-aged adults independently of potential confounders such us age, cardiorespiratory fitness, physical activity levels and dietary intake.

SECTION 3: Role of exercise on S-Klotho protein, physical fitness, energy metabolism and cardiometabolic health

- Exercise training induces an increase of S-Klotho in sedentary middle-aged adults. Moreover, changes in body composition are related to changes in S-Klotho after an exercise training programme.
- A 12-week HIIT program adding or not WB-EMS, as well as a PAR program, can be used as a strategy to improve body composition obtaining slightly better results with the application of a HIIT+EMS intervention in sedentary middle-aged adults.
- A 12-week structured exercise intervention improves physical fitness regardless of the training exercise modality in sedentary middle-aged adults.
- A 12 week-long, HIIT+EMS program improves BMR, BFox, and MFO in sedentary middle-aged adults. Moreover, these improvements were clinically better than those induced by a 12 week-

long HIIT without WB-EMS, and a 12-week PAR program for sedentary middle-aged adults.

- A 12-week HIIT program combined with WB-EMS can significantly reduce cardiometabolic risk in sedentary, middle-aged adults independent of sex, age and cardiorespiratory fitness. Further, exercise-induced changes in LM seem to be a powerful predictor of improvements in cardiometabolic risk after a training intervention.

FUTURE PERSPECTIVES

FUTURE PERSPECTIVES

- Future intervention studies applying similar exercise protocols in younger/older healthy people and patients with metabolic disorders (i.e. type II diabetes mellitus) are needed to investigate whether these findings are applicable for individuals with different biological characteristics.
- Given that time-restricted feeding is a well-known anti-ageing strategy, further studies are necessary to investigate whether the combination of new training methodologies (i.e. HIIT+EMS) and time-restricted feeding provides extra benefits on human health during the ageing process.
- The findings of the present International Doctoral Thesis suggest that HIIT+EMS provides independent and additive benefits in S-Klotho, physical fitness, energy metabolism and cardiometabolic risk. However, future studies should test whether a PAR intervention plus WB-EMS induces similar or even greater effects on human health compared with a HIIT+EMS intervention.
- Future studies should measure the α-Klotho, β-Klotho and γ-Klotho gene expresion in response to exercise, as well as the intra-cellular form and the cell-membrane form of the α-Klotho gene, which would have allowed to better understand the role of exercise on the Klotho gene regulation. In addition, it is necessary to investigate whether S-Klotho changes induced by chronic exercise could be mediated by changes in IGF-I, TGF-β, Wnt signalling, and IFNγ, since these parameters were not measured in our study.
- Future investigations should focus on the study of transcriptomic, metabolomic, lipidomic and proteomic in response to the above-described exercise interventions, in order to well-understand the physiological processes that mediates exercise-related benefits on human health.

ANEXES

Short- Curriculum Vitae

Publications

Papers derived from the International Doctoral Thesis

1 **Amaro Gahete FJ,** De La O A, Jurado Fasoli L, Castillo MJ, Gutierrez A. Fitness Assessment as an Anti-Aging Marker: A Narrative Review. *J Gerontol Geriatr Res* 2017; **06** (6): 455.

2 **Amaro-Gahete FJ**, de la O A, Jurado-Fasoli L, Ruiz JR, Castillo MJ, Gutierrez A. Role of exercise on S-Klotho protein regulation: a systematic review. *Curr Aging Sci* 2018; **11** (2): 100-107**.**

3 **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**.**

4 **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. In press.

5 **Amaro-Gahete FJ**, Sanchez-Delgado G, Alcantara JMA, Martinez-Tellez B, Acosta FM, Helge JW *et al.* Impact of data analysis methods for maximal fat oxidation estimation during exercise in sedentary adults. *Eur J Sport Sci* 2019; **28**: 1–10.

6 **Amaro-Gahete FJ**, Sanchez-Delgado G, Helge JW, Ruiz J. Optimizing maximal fat oxidation assessment by a treadmill-based graded exercise protocol: when should the test end? *Submitted*.

7 **Amaro-Gahete FJ,** Sanchez-Delgado G, Ruiz JR. Commentary: Contextualising Maximal Fat Oxidation During Exercise: Determinants and Normative Values. *Front Physiol* 2018; **9**: 1460.

8 **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; **31**: 1–20.

9 **Amaro-Gahete FJ**, 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.

10 **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. In press.

11 **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 sedentary middle-aged adults: the FIT-AGEING study. *Maturitas* 2019; **123**: 25-31.

12 **Amaro-Gahete FJ**, De-la-O A, Jurado-Fasoli, Ruiz J, Castillo M. Association of basal metabolic rate and fuel oxidation in basal conditions and during exercise, with plasma S-Klotho. *Submitted*.

13 **Amaro-Gahete FJ,** Jurado-Fasoli L, De-la-O A, Ruiz J, Castillo M. Relationship of S-Klotho and cardiometabolic risk in sedentary middle-aged adults: the FIT-AGEING study. *Submitted*.

14 **Amaro-Gahete FJ**, De-la-O A, Jurado-Fasoli L, Espuch-Oliver A, de Haro T, Gutiérrez A *et al*. Exercise training increases the S-Klotho plasma levels in sedentary middle-aged adults: a randomised controlled trial. *J Sport Sci* 2019. In press.

15 **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. In press.

16 **Amaro-Gahete FJ**, De-la-O A, Jurado-Fasoli L, Dote-Montero M, Gutiérrez A, Ruiz J *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 randomised controlled trial. *Front Physiol* 2019. In press.

17 **Amaro-Gahete FJ,** De-la-O A, Jurado-Fasoli L, Sanchez-Delgado G, Ruiz J, Castillo M. Basal metabolic rate and fat oxidation in basal conditions and during exercise in sedentary middle-aged adults, following different exercise training interventions: a randomised controlled trial THE FIT AGEING Study. *Submitted*.

18 **Amaro-Gahete FJ**, De-la-O A, Jurado-Fasoli L, Martinez-Tellez B, Ruiz J, Castillo J. Exercise training as a treatment for cardiometabolic risk in sedentary middle-aged adults: are physical activity guidelines the best way to improve cardiometabolic health? The FIT-AGEING randomized controlled trial. *Submitted*.

Others papers

19. **<u>Amaro-Gahete FJ</u>**, de la O A, Jurado-Fasoli L, Ruiz JR, Gutiérrez Á. 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.

20. **<u>Amaro-Gahete FJ</u>**, 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.

21. **<u>Amaro-Gahete FJ</u>**, De-la-O A, Robles-González L, Castillo M, Gutierrez A. Impact of two whole-body electromyostimulation training modalities on body composition in recreational runners during endurance training cessation. *Rev Int Cienc Deporte* 2018; **53**: 205–218.

22. **<u>Amaro-Gahete FJ</u>**, 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.

23. **<u>Amaro-Gahete FJ</u>**, Ruiz JR. Methodological issues related to maximal fat oxidation rate during exercise : Comment on: Change in maximal fat oxidation in response to different regimes of periodized high-intensity interval training (HIIT). *Eur J Appl Physiol* 2018; **118**: 2029–2031.

24. **<u>Amaro-Gahete FJ</u>**, Sanchez-Delgado G, Alcantara JMA, Martinez-Tellez B, Muñoz-Hernandez V, Merchan-Ramirez E *et al.* Congruent Validity of Resting Energy Expenditure Predictive Equations in Young Adults. *Nutrients* 2019; **11**: 1–13 ***(IF: 4.169)***.

25. **<u>Amaro-Gahete FJ</u>**, Sanchez-Delgado G, Alcantara JMA, Martinez-Tellez B, Acosta F, Merchan-Ramirez E *et al.* Energy expenditure differences across lying, sitting, and standing positions in young healthy adults. *PLoS One* 2019. In press.

26. Dote-Montero M[ϕ], **<u>Amaro-Gahete FJ[ϕ]</u>**, 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.

27. Jurado-Fasoli L, **<u>Amaro-Gahete FJ</u>**, De-la-O A, Dote-Montero M, Gutiérrez Á, Castillo MJ. Association between Sleep Quality and Body Composition in Sedentary Middle-

Aged Adults. *Medicina (Kaunas)* 2018; **54** (5): E91**.**

28. Jurado-Fasoli L, **Amaro-Gahete FJ**, De-la-O A, Gutiérrez Á, Castillo MJ. Alcohol consumption and S-Klotho plasma levels in sedentary healthy middle-aged adults: A cross sectional study. *Drug Alcohol Depend* 2019; **194**: 107–111 **.**

29 Jurado-Fasoli L, **Amaro-Gahete FJ**, De-la-O A, Martinez-Tellez B, Ruiz JR, Gutiérrez Á *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.

30. Acosta FM, Martinez-Tellez B, Sanchez-Delgado G, Migueles JH, Contreras-Gomez MA, Martinez-Avila WD, […], **Amaro-Gahete FJ**, *et al.* Association of Objectively Measured Physical Activity With Brown Adipose Tissue Volume and Activity in Young Adults. *J Clin Endocrinol Metab* 2019; **104**: 223–233.

31. Molina-Hidalgo C$^{\phi}$, De-la-O A$^{\phi}$, Jurado-Fasoli. L, Amaro-Gahete F, Castillo M. Beer or Ethanol Effects on the Body Composition Response to High-Intensity Interval Training. The BEER-HIIT Study. *Nutrients* 2019; **11**: 909.

32. Arouca A, Moreno LA, Gonzalez-Gil EM, Marcos A, Widhalm K, Molnár D, […], **Amaro-Gahete FJ**, *et al.* Diet as moderator in the association of adiposity with inflammatory biomarkers among adolescents in the HELENA study. *Eur J Nutr* 2018; **107**: 1-14.

33. González Gil E, Gracia-Marco L, Santabárbara J, Molnar D, **Amaro Gahete FJ**, Gottrand F *et al.* Inflammation and insulin resistance according to body composition in European adolescents: the HELENA study. *Nutr Hosp* 2017; **34** (5): 1033-1043**.**

34. González-Gil EM, Cadenas-Sanchez C, Santabárbara J, Bueno-Lozano G, Iglesia I, González-Gross M, […], **Amaro-Gahete FJ**, *et al.* Inflammation in metabolically healthy and metabolically abnormal adolescents: The HELENA study. *Nutr Metab Cardiovasc Dis* 2018; **28**: 77–83**.**

35. Gutiérrez-Dávila M, Garrido J, **Amaro-Gahete FJ**, Ramos M, Rojas F. Método para determinar la contribución segmentaria en los saltos. Su aplicación en el salto vertical con contramovimiento. *Mot Eur J Hum Movement* 2012; **29**: 1–16.

36. Gutiérrez-Dávila M, **Amaro-Gahete FJ**, Garrido JM, Javier Rojas F. An analysis of two styles of arm action in the vertical countermovement jump. *Sport Biomech* 2014; **13**: 135–143.

37. Gutiérrez-Dávila M, Garrido JM, **Amaro-Gahete FJ**, Rojas FJ. Contribución segmentaria de los saltos con contramovimiento en vertical y en horizontal. [Segmental contribution on countermovement vertical and horizontal jumps]. *RICYDE Rev Int ciencias del Deport* 2014; **10**: 289–304 **.**

38. Martinez-Tellez B, Sanchez-Delgado G, Alcantara JMA, Acosta FM, **Amaro-Gahete FJ**, Osuna-Prieto FJ *et al.* Evidence of high 18 F-fluorodeoxyglucose uptake in the subcutaneous adipose tissue of the dorsocervical area in young adults. *Exp Physiol* 2019; **104**: 168–173.

39. Sanchez-Delgado G, Alcantara JMA, Acosta FM, Martinez-Tellez B, **Amaro-Gahete FJ**, Ortiz-Alvarez L *et al.* Estimation of non-shivering thermogenesis and cold-induced nutrient oxidation rates: Impact of method for data selection and analysis. *Clin Nutr* 2018. In press**.**

40. Sanchez-Delgado G, Martinez-Tellez B, Garcia-Rivero Y, Acosta FM, Alcantara JMA, **Amaro-Gahete FJ** *et al.* Association between brown adipose tissue and bone mineral density in humans. *Int J Obes (Lond)* 2018. In press**.**

41. Sanchez-Delgado G, Martinez-Tellez B, Garcia-Rivero Y, Alcantara JMA, Acosta FM, **Amaro-Gahete FJ** *et al.* Brown Adipose Tissue and Skeletal Muscle 18F-FDG Activity After a Personalized Cold Exposure Is Not Associated With Cold-Induced Thermogenesis and Nutrient Oxidation Rates in Young Healthy Adults. *Front Physiol* 2018; **9**: 1577.

42. Sanchez-Delgado G, Martinez-Tellez B, Olza J, Aguilera CM, Labayen I, Ortega FB […], **Amaro-Gahete FJ**, *et al.* Activating brown adipose tissue through exercise (ACTIBATE) in young adults: Rationale, design and methodology. *Contemp Clin Trials* 2015; **45**: 416–425.

43. Martinez-Tellez B, Garcia-Rivero Y, Sanchez-Delgado G, Xu H, **Amaro-Gahete FJ,** Acosta FM *et al.* Supraclavicular skin temperature measured by iButtons and 18F-fluorodeoxyglucose uptake by brown adipose tissue in adults. *J Therm Biol* 2019; **82**: 178–185.

44. Martinez-Tellez B, Perez-Bey A, Sanchez-Delgado G, Acosta FM, Corral-Perez J, **Amaro-Gahete FJ** *et al.* Concurrent validity of supraclavicular skin temperature measured with iButtons and infrared thermography as a surrogate marker of brown adipose tissue. *J Therm Biol* 2019; **82**: 186–196.

45. Jurado-Fasoli L, **Amaro-Gahete FJ,** De-la-O A, Castillo M. Impact of different exercise training modalities on energy and nutrients intake and food consumption in sedentary middle-aged adults: a randomized controlled trial. *J Hum Nutr Diet* 2019. In press.

$^{\phi}$ Equally contributed

Invited conferences

1. **Amaro-Gahete FJ**. Whole-body electromyostimulation training and health biomarkers during the ageing process. I Congress EMS Training: Safety, Health and Sport Performance. Milan, Italy. 1st December, 2018.
2. **Amaro-Gahete FJ**. Functional and periodized whole-body electromyostimulation training on Running Performance in detraining phases. II WB-EMS International Simposium. Lisboa, Portugal. 7th October, 2018
3. **Amaro-Gahete FJ**. Whole-Body electromyostimulation and High Intensity Interval Training. I WB-EMS International Simposium. Porto, Portugal. 22nd October, 2017.
4. **Amaro-Gahete FJ**. Entrenamiento con Electroestimulación Global: Eficiencia y Economía de Carrera en corredores. II Congreso International de electroestimulación integral. Madrid, Spain. 16th April, 2016.

Other merits

2012- Co-author of more tan 30 congress communications (including national and international conferences).

2014 Certified personal trainer. National Strength and Conditionining Association.

2016- Lecturer in the degree of Medicine. University of Granada.

2016- Lecturer in the degree of Sports Sciences. University of Granada.

2016- Lecturer in the degree of Phyiotherapy. University of Granada.

2018- Lecturer in the master degree in Food, Exercise and Sports for Health. University of Granada.

2018- Reviewer of several scientific indexed journal: *Frontiers in Physiology, Osteoporosis International, BMC Public Health,* and *Journal of Aging and Physical Activity.*