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Regulación de los sistemas antioxidantes musculares por ejercicio físico y dieta en seres humanos: implicaciones para el desarrollo de un producto turístico de alto valor añadido para Canarias

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Tesis Doctoral

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Presentaciones

Ponencia 1: Defensa oral para el concurso YIA. Presentado por Víctor Galván Álvarez "Anthropometric and body composition equations to predict resting energy expenditure in overweight and obese men and women living in a temperate climate". http://hdl.handle.net/10553/119851

Ponencia 2: Defensa oral para el concurso YIA. Presentado por Víctor Galván Álvarez "Accurate assessment of energy expenditure in the main seafront walking route of Las Palmas de Gran Canaria to promote health related tourism". https://www.ecss2006.com/asp/congress/TOOLS/Benefits/EDSS_Results.asp

Ponencia 3: "Aerobic fitness, but not age and sex, determines the levels of antioxidant enzymes and its main upstream regulatory transcription factors nrf2/keap1 in human skeletal muscle". https://www.ecss2006.com/asp/congress/TOOLS/Benefits/EDSS_Results.asp

Ponecia 4 y 5: de "International Symposium on Updates in Physical Activity, Health and Sport Performance and Scientific Conference of the Research Network In Motu Salus" exponiendo resultados de la investigación:

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LISTA DE ABREVIATURAS

ARE, elementos de respuesta a antioxidantes

CSQ, calsecuestrina

DM2, diabetes mellitus tipo 2

GR, glutatión reductasa

IMC, índice de masa corporal

Keap1, kelch-like ECH-associated protein 1

LLM, lower extremities lean mass - masa magra de las extremidades inferiores

MHC I, myosin heavy chain type I

MHC IIa, myosin heavy chain type IIa

MHC IIx, myosin heavy chain type IIx

MHC, myosin heavy chain – cadenas pesadas de miosinas

NF-κB p65, nuclear factor NF-kappa-B p65 subunit

Nrf2, nuclear factor (erythroid-derived 2)-like 2

P38 MAPK, p38 mitogen-activated protein kinase

PFKM, fosfofructoquinasa

RF, reserva funcional

RNS, especies reactivas del nitrógeno

RONS, especies reactivas de oxígeno y nitrógeno

ROS, especies reactivas del oxígeno

SDHB, NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8

SERCA, ATPasa de calcio del retículo sarcoplásmico

SODs, superoxide dismutase

VO_2max, consumo máximo de oxígeno

WAPI, puntos de interés para caminantes

Wmean, potencia media durante la prueba de Wingate

Wpeak, potencia máxima instantánea durante la prueba de Wingate

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RESUMEN

RESUMEN

Introducción

El sedentarismo y la obesidad son dos de los principales problemas de salud a los que se enfrentan las sociedades modernas, incluida Canarias. La obesidad en particular se asocia a un incremento marcado de la prevalencia de al menos 13 tipos de cáncer, artrosis, diabetes mellitus tipo 2, enfermedad cardiovascular y neurodegenerativa, deteriora la calidad de vida y acorta la expectativa de vida. Uno de los principales mecanismos patogénicos a través de los cuales se producen y cursan estas enfermedades es el estrés oxidativo.

Tanto el ejercicio físico como la restricción calórica se asocian a un aumento transitorio de la producción de radicales libres y, en ocasiones, a estrés oxidativo. Este aumento de la producción de radicales libres provoca una respuesta contrarreguladora que conduce a un aumento de la capacidad antioxidante y adaptaciones celulares provocadas por las especies reactivas de oxígeno y nitrógeno (Reactive Oxygen and Nitrogen Species - RONS), que mejoran la capacidad y eficacia antioxidante. Se desconoce, no obstante, cual es el mejor modelo de ejercicio para provocar un aumento más eficaz de los sistemas antioxidantes. Tampoco se sabe cómo interacciona el ejercicio en la inducción de los sistemas antioxidantes musculares en seres humanos. Además, los escasos estudios acerca de los efectos del ejercicio se han realizado mayormente en varones, por lo que es necesario llevar a cabo nuevos estudios que incluyan a mujeres.

La presente tesis doctoral tiene como objetivo central investigar la importancia del sistema antioxidante en la musculatura esquelética en seres humanos, y su regulación a través de diferentes modelos de ejercicio físico. El tejido muscular esquelético es fundamental para la salud debido a su función en el movimiento, el control de la glucosa y la sensibilidad a la insulina, su contribución al rendimiento y función física y su influencia en la salud cardiovascular y a través del ejercicio y la liberación de factores metabólicos y hormonas (mioquinas). A partir de la información obtenida, esta investigación busca impulsar la creación de productos turísticos innovadores orientados al turismo de salud que contribuyan al desarrollo regional de Canarias.

Para llevarlo a cabo, en primer lugar, se ha examinado la expresión basal de proteínas reguladoras de la capacidad antioxidante en el músculo esquelético humano en más de 200 seres humanos con amplio rango de edades, incluyendo personas jóvenes y mayores, hombres y mujeres y diferentes niveles de condición física, hábitos de vida y adiposidad. El estudio de la regulación de este sistema antioxidante a través de ejercicio físico se ha llevado a cabo utilizando varios modelos de ejercicio físico agudo.

Los resultados muestran que, al considerar las diferencias de sexo en VO_2max (el predictor más potente de la esperanza de vida), edad y adiposidad, la expresión proteica de Nrf2 (nuclear factor (erythroid-derived 2)-like 2) y las principales enzimas antioxidantes es similar en hombres y mujeres. Además, se ha demostrado que un mayor VO_2max se asocia con una mayor expresión basal de Nrf2 total, Keap1 y SOD2 (superoxide dismutase 2) en el músculo esquelético humano.

Seguidamente, hemos analizados los factores que determinan el rendimiento durante el ejercicio de alta intensidad repetido, usando un nuevo modelo desarrollado en nuestro laboratorio, que permite determinar la Reserva Funcional (RF) La RF es la capacidad para generar energía a niveles iguales o superiores a los alcanzados en el momento del agotamiento. Nuestros resultados indican que la reserva funcional depende en parte de la expresión de enzimas antioxidantes y sus factores reguladores. Además, hemos cuantificado por primera vez la magnitud de esta RF. Para lo cual se llevaron a cabo series repetidas supramáximas al 120% de VO_2max, intercaladas con periodos de recuperación de 20 segundos con isquemia completa. Se involucraron 43 adultos jóvenes y sanos, 30 de ellos hombres.

Además, identificamos factores musculares clave que afectan el rendimiento en sprint. Se midieron parámetros relacionados con la composición muscular y el rendimiento en adultos jóvenes físicamente activos (51 hombres y 10 mujeres). A pesar de las diferencias de género en algunas proteínas musculares, ambos sexos presentaron un rendimiento en sprint similar cuando se normalizó según la masa magra de las piernas (lower extremities lean mass - LLM). Este estudió ha demostrado que la potencia máxima en el sprint depende de la expresión de ciertas proteínas, como SDHB (NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8) y Keap1 (kelch-like ECH-

associated protein 1), junto con el tipo de isoforma de las cadenas pesadas de miosina (MHC) que predomina en el vasto lateral del cuádriceps.

Finalmente, los datos presentados en esta tesis, así como otros presentados en congresos obtenidos en 15 voluntarios que caminaron durante 4 días unas 8 horas diarias mientras estaban sometidos a una dieta de 330 Kcal/d, indican que tanto el ejercicio de alta intensidad como el ejercicio prolongado de baja intensidad activan la expresión de enzimas antioxidantes en los músculos esqueléticos. La tesis incluye un estudio adicional en el que se determina el gasto energético de marcha en personas con composición corporal normal o sobrepeso u obesidad en el paseo de las Canteras. Los datos recogidos indican que este tipo de actividad es factible y bien tolerado, por lo que puede utilizarse para inducir cambios favorables en la capacidad antioxidante de los músculos esqueléticos, al tiempo que minimiza el riesgo de lesión en las personas mayores o que tienen sobrepeso u obesidad. La ruta analizada es ideal para generar un producto orientado al turismo de salud, por reunir la mayoría de los requisitos que debe tener una ruta urbana para ser atractiva a los turistas como son: la caminabilidad, el atractivo paisajístico, la buena iluminación, la cercanía del mar, la limpieza del entorno y la seguridad ciudadana. En esta última parte de la tesis se ha cuantificado el gasto energético en cada punto kilométrico de la ruta, lo que permite generan carteles con indicaciones para los usuarios, que les permitan conocer el coste energético de cada tramo.

Material y métodos

En el marco de la presente tesis doctoral, se llevaron a cabo cuatro estudios. Para ello, se aplicaron diferentes métodos y técnicas de investigación en una muestra heterogénea de participantes.

Estudio 1: Se centró en la determinación de los niveles de expresión de enzimas antioxidantes (SOD1, SOD2, catalasa y glutatión reductasa) y el principal eje regulador del sistema antioxidante (Nrf2/Keap1) en una muestra de 189 voluntarios, compuesta por 120 hombres y 69 mujeres. El objetivo era establecer si existían diferencias entre sexos y determinar si la edad, el VO_2max y la adiposidad influyen en la expresión proteica de enzimas antioxidantes y sus principales sistemas reguladores en el músculo esquelético

humano. Para llevar a cabo esta investigación, se obtuvieron biopsias musculares del vasto lateral en condiciones de reposo y sin estrés.

Estudio 2: Este estudio se enfocó en la determinación de la RF. Para ello, se llevaron a cabo series repetidas de ejercicio supramáximo al 120% de VO_2max, con intervalos de recuperación de 20 segundos en isquemia completa. La muestra incluyó 43 adultos jóvenes y sanos, entre ellos 30 hombres y 13 mujeres. Durante la investigación, se evaluaron el rendimiento físico, las variables cardiorrespiratorias, el déficit de oxígeno y la oxigenación cerebral y muscular a través de la espectroscopia cercana al infrarrojo. Además, se obtuvieron biopsias musculares en reposo de todos los participantes.

Estudio 3: Se centró en investigar los principales determinantes fisiológicos del rendimiento en el ejercicio de sprint en cicloergómetro en adultos jóvenes físicamente activos, con particular énfasis en el papel de los RONS y los principales determinantes fenotípicos de vasto lateral del cuádriceps: porcentaje de MHC I, IIa y IIx, proteínas reguladoras de la concentración sarcoplasmática de calcio, así como enzimas del metabolismo aeróbico y glucolítico. La muestra consistió en 51 hombres y 10 mujeres. Para la medición de la composición corporal, se utilizó la absorciometría fotónica de rayos X de doble energía. Se realizaron pruebas de sprint con el test isocinético de 30 segundos de Wingate, evaluando la potencia pico y promedio (Wpeaki y Wmean). Asimismo, se obtuvo una biopsia muscular en reposo del músculo vasto lateral.

Estudio 4: Se abordó el análisis del gasto energético en la ruta principal de Las Palmas de Gran Canaria, el paseo de las Canteras, que abarca un recorrido total de 13 kilómetros (ida y vuelta). La evaluación se realizó en treinta y tres voluntarios, compuestos por 22 hombres y 11 mujeres, con variados niveles de adiposidad y amplio rango de edad y adiposidad. Para determinar el gasto energético, se utilizó un metabolímetro portátil (COSMED K5), y se empleó la geolocalización para identificar el consumo de energía correspondiente a cada sector kilométrico específico. Además, se generaron datos para ser usado en paneles informativos para los caminantes, con el propósito de fomentar el turismo de salud.

Resultados

El estudio 1, indica que al considerar las diferencias de género en VO_2max, edad y adiposidad, el Nrf2 y las principales enzimas antioxidantes en el músculo esquelético humano se expresan en cantidades similares en hombres y mujeres. Además, hemos demostrado que una mejor condición aeróbica (es decir, un VO_2max más alto por kg de masa magra de las extremidades inferiores) se relaciona con una mayor expresión basal de Nrf2 total, Keap1 y SOD2. Nuestros resultados también indican que SOD2 es el mejor predictor de VO_2max en el músculo esquelético humano, mientras que la expresión de SOD1 aumenta con la edad después de tener en cuenta las diferencias en VO_2max y el porcentaje de grasa corporal. Por último, hemos mostrado que un IMC $\geq$ 25 kg/m² se asocia con niveles elevados de pSer^{40}-Nrf2, la relación pSer^{40}-Nrf2/Nrf2 total y los niveles de expresión de SOD1, después de ajustar por las diferencias en edad y VO_2max normalizado a la masa magra de las extremidades inferiores.

El estudio 2 ha demostrado que la RF es similar en hombres y mujeres jóvenes, físicamente activos, cuando se normalizó en relación con la LLM. La RF depende el VO_2 acumulado (94.1%), y de manera secundaria el déficit de O_2, la potencia media del test y el porcentaje de MHCI+IIa en el vasto lateral del cuádriceps. El componente aeróbico está relacionado con la oxigenación cerebral y ciertas características fenotípicas, implicadas en la regulación del calcio (SERCA1 y SERCA2), el transporte y la difusión de oxígeno (mioglobina) y la regulación redox (Keap1). El componente glucolítico se puede predecir mediante la medición de los niveles de expresión de proteínas como SOD1 y pSer^{40}-Nrf2, y el déficit acumulado máximo de oxígeno. Hemos demostrado que la RF tiene una magnitud finita que oscila entre 0.17-3.66 kJ. kg LLM^{-1} en humanos jóvenes sanos.

El estudio 3, ha demostrado que el principal predictor de rendimiento de sprint en cicloergómetro fue la LLM, aunque las mujeres mostraron un mayor porcentaje de MHC I, SERCA2, pSer^{16}/Thr^{17}-fosfolamban y expresión de proteína de Calsecuestrina-2 (CSQ-2) (todos $P < 0.05$), y un 18.4% menos de expresión de la proteína fosfofructoquinasa-1 (PFKM) que los hombres ($P < 0.05$), ambos sexos presentaron un rendimiento en sprint similar cuando se normalizó por LLM. El análisis de regresión múltiple reveló que Wpeaki se podía predecir a partir de LLM, SDHB, Keap1 y el porcentaje de MHC II

(R^2=0.62, P <0.001), explicando las variables anteriores el 46.4, 6.3, 4.4 y 4.3% de la varianza en Wpeaki, respectivamente. La LLM y el porcentaje de MHC II explicaron el 67.5 y 2.1% de la varianza en Wmean, respectivamente (R^2=0.70, P <0.001). Así mismo, este estudio ha constatado que SDHB y Keap1, junto con el porcentaje de MHC II, son determinantes relevantes de la potencia pico durante el sprint.

Globalmente nuestros datos de este estudio indican que el ejercicio físico es clave para potenciar la capacidad antioxidante del organismo. Así mismo, hemos demostrado que el sobrepeso y la obesidad se asocian a un aumento de la expresión proteica de algunas enzimas antioxidantes, al igual que ocurre con el envejecimiento. Por lo tanto, el siguiente paso fue proponer un modelo de ejercicio que permitiera aplicar estos conocimientos y que se pudiera beneficiar a la población general, pero también ser utilizado para atraer turistas a Canarias, a través de una cuantificación exacta del gasto energético que genera el ejercicio, en un entorno de alto valor turístico.

El estudio 4 ha permitido cuantificar cuántas calorías se queman por km recorrido en la principal ruta urbana para caminar de Gran Canaria. Hemos generado información útil para los interesados, que se puede emplear en establecer "puntos de interés para caminantes" (WAPI) donde se reflejen las kcal quemadas por kilómetro recorrido, así como otra información de interés turístico. Además, hemos observado que el gasto energético en caminatas largas está influenciado principalmente por el peso corporal y la distancia recorrida, con un aumento leve del gasto energético a medida que se aumenta la distancia, que llega a ser un 9% mayor en el km 13 que, en el km inicial, demostrando que la duración de la caminata per se aumenta el gasto energético.

Conclusiones

El VO_2max se asocia con una mayor expresión basal del eje regulador del sistema antioxidante, Nrf2/Keap1, así como de la enzima SOD2 en el músculo esquelético humano. Las principales enzimas antioxidantes y el Nrf2 se expresan de forma similar en el músculo esquelético humano de ambos sexos cuando se tienen en cuenta las diferencias en VO_2max, edad y adiposidad. Además, nuestros estudios han revelado que tanto una sesión de ejercicio intenso como el entrenamiento, ya sea de baja o alta intensidad, provocan una regulación al alza de la capacidad antioxidante en el tejido muscular.

Por otro lado, hemos demostrado que los principales factores que determinan el rendimiento en sprint en el ergómetro son la LLM y el porcentaje de MHCII, mientras que SDHB y Keap1 contribuyen en menor medida.

Finalmente, hemos cuantificado el gasto energético de la caminata prolongada en llano y hemos propuesto una iniciativa innovadora orientada a potenciar los sistemas antioxidantes musculares a través de la promoción de rutas a pie en escenarios turísticos de fácil acceso y alta caminabilidad en Canarias. La información precisa sobre el gasto de energía que hemos recopilado se utilizará para crear puntos de interés turístico diseñados específicamente para fomentar el turismo de salud.

II

INTRODUCCIÓN

INTRODUCCIÓN

El sedentarismo y la obesidad son dos de los principales problemas de salud a los que se enfrentan las sociedades modernas [1], incluida Canarias [2, 3]. Al menos 13 tipos de cáncer [4, 5], la artrosis, la diabetes mellitus tipo 2, la coronariopatía isquémica, la litiasis biliar, la hipertensión arterial y la insuficiencia renal crónica [6-9], tienen mayor prevalencia y peor evolución en las personas con obesidad. La obesidad es responsable de al menos el 9% del gasto sanitario en USA [10], deteriora la calidad de vida de los pacientes [11] y acorta la expectativa de vida [10]. La inactividad física es un factor de riesgo independiente de la obesidad, especialmente importante en personas mayores, que se ha asociado al riesgo de caídas y enfermedad de Alzheimer y otras enfermedades neurodegenerativas [12]. Numerosos estudios epidemiológicos, intervenciones y ensayos clínicos aleatorizados han demostrado que el tratamiento más eficaz para la obesidad y la inactividad física es el ejercicio físico combinado con dieta hipocalórica [13].

Uno de los principales mecanismos patogénicos a través de los cuales se producen y cursan las principales enfermedades crónicas, incluida la obesidad y la diabetes mellitus tipo 2, son la inflamación y el estrés oxidativo. Los radicales libres son átomos o moléculas que contienen uno o más electrones desapareados que son capaces de existencia independiente. Los radicales libres pueden tener una existencia muy breve debido a su alta reactividad con otras moléculas. Las especies reactivas del oxígeno (ROS) son compuestos (o átomos) que contienen oxígeno, que pueden ser radicales libres o derivados de oxígeno altamente reactivos no radicales, como el peróxido de hidrógeno y otros. Las especies reactivas del nitrógeno (RNS) son radicales de nitrógeno y moléculas reactivas de nitrógeno no radicales con un centro reactivo de nitrógeno [14]. El término especies reactivas de oxígeno y nitrógeno (RONS) se usa a menudo para referirse indistintamente a ROS y RNS.

Los radicales libres fueron identificados por primera vez en materiales biológicos en 1954 [15]. La alta reactividad de los radicales libres facilita su interacción con biomoléculas causando en algunos casos modificaciones irreversibles que conducen a la pérdida de la función y al daño celular. En consecuencia, surgió un concepto predominante considerando los radicales libres como "agentes peligrosos" que deberían

evitarse y contrarrestarse [16]. De hecho, los RONS están implicados en múltiples mecanismos de enfermedad que incluyen inflamación, cáncer [17], mutagénesis, lesión inducida por radiación [18], enfermedades neurodegenerativas [19] y envejecimiento [20-22]. Al mismo tiempo, los estudios epidemiológicos informaron una asociación inversa entre la ingesta de alimentos ricos en antioxidantes (α- tocoferol, ácido ascórbico y β-caroteno) y el riesgo de cáncer y enfermedades cardiovasculares [23]. Asimismo, la ingestión de alimentos ricos en polifenoles se ha asociado con un menor riesgo cardiovascular, cerebrovascular y de enfermedad vascular periférica [24] y una menor incidencia de algunos cánceres [25, 26]. En consecuencia, durante los últimos años de la década de los 80 y los años siguientes, se hizo hincapié en estudiar el papel de los antioxidantes para prevenir y tratar enfermedades, así como en la terapia de rejuvenecimiento. Inesperadamente, la mayoría de los ensayos clínicos con antioxidantes no mostraron ningún beneficio para la salud [27-29], se ha publicado que la suplementación con β-caroteno, vitamina A y E [30] o vitamina C [31] se asocia con una mayor mortalidad.

En la década de 1930, Warburg y Szent-Gyorgyi formularon la hipótesis de que las reacciones redox tienen un papel en la regulación de las funciones celulares [32]. En la década de los 70 la tecnología permitió demostrar que las RONS son necesarias para el funcionamiento normal de las células. En ese período, se observó que las ratas sometidas a hiperoxia en 100% de oxígeno (1 atmósfera) mueren al cabo de 3 días. Sin embargo, las ratas pre-expuestas a 80% de oxígeno durante 5 días, sobrevivieron y se adaptaron al alto ambiente de O_2 de tal manera que si se exponían a una atmosfera con un 100% de oxígeno podía sobrevivir indefinidamente [32]. Por lo tanto, quedó claro que las células y los organismos vivos pueden adaptarse al estrés oxidativo subletal aumentando la tolerancia a las exposiciones subsiguientes a RONS. Además, se descubrió que múltiples funciones fisiológicas requieren radicales libres o sus derivados, incluyendo la regulación del tono vascular, la quimio-detección de O_2, varios mecanismos de transducción de señales y respuestas adaptativas al estrés oxidativo necesarias para el mantenimiento de la homeostasis redox y la salud [33, 34]. Las RONS también son necesarias para el plegamiento normal de proteínas y la formación de enlaces disulfuro que regulan la estructura y la función de muchas proteínas celulares [35].

Las RONS existen en células y tejidos a bajas concentraciones, dependiendo del equilibrio entre sus tasas de producción y sus tasas de eliminación por los compuestos antioxidantes y las enzimas quelantes de RONS (o enzimas antioxidantes) [34]. El término estrés oxidativo fue acuñado por Helmut Sies [36] para referirse a una alteración del equilibrio redox. El estrés oxidativo puede causar daño oxidativo de la mayoría de las biomoléculas [34], particularmente aquellas que están más cerca de la fuente de RONS, como es el caso de las biomoléculas mitocondriales. Cuando el equilibrio redox se altera por el aumento de la producción de RONS o la disminución de la capacidad antioxidante, esto provoca un desplazamiento temporal del equilibrio redox tiol / disulfuro hacia una condición más oxidativa. Esto desencadena la señalización redox que provoca respuestas contrarreguladoras para restablecer la homeostasis redox [34]. En ciertas circunstancias, como el envejecimiento y algunas condiciones patológicas, las respuestas contrarreguladoras pueden cambiar el nivel de homeostasis a un estado oxidativo más alto. En consecuencia, el daño oxidativo puede surgir de la alteración directa de biomoléculas por RONS y por los cambios provocados por la señalización en la expresión génica [34]. La homeostasis redox se mantiene gracias a los antioxidantes y la modulación de las fuentes de producción de RONS y la capacidad antioxidante. Los antioxidantes son compuestos que a concentraciones relativamente bajas compiten con otros sustratos oxidables retrasando o evitando su oxidación [34]. Los antioxidantes se pueden dividir en dos categorías: antioxidantes enzimáticos y no enzimáticos. Las enzimas antioxidantes más importantes en el músculo esquelético son la SODs, glutatión peroxidasa (GPx) y catalasa [34].

La activación del factor 2 relacionado con el factor eritroide nuclear 2 (Nrf2) está mediada por el estrés oxidativo y constituye el eje central de la regulación antioxidante en las células del organismo [37]. En condiciones de homeostasis normal hay poco Nrf2 en el citoplasma, ya que el Nrf2 que se va formando se dirige de manera constitutiva a la degradación proteasomal mediante ubiquitinación [38]. La inducción de Nrf2 se activa al inhibir la ubiquitinación de Nrf2 dependiente de KEAP1 [39]. En condiciones basales, Keap1 se une a Nrf2 en el citoplasma y promueve su ubiquitinación por la ligasa ubiquitina E3 que contiene Cul3, lo que dirige a Nrf2 a la degradación proteasomal [40]. El sistema Nrf2-Keap1 es un sistema de defensa destinado a preservar la homeostasis celular [41]. La interrupción de la señalización Nrf2-Keap1 puede ocurrir durante el

envejecimiento humano y contribuir a la sarcopenia [42]. Cuando la cantidad de Keap1 libre es baja, el Nrf2 recién sintetizado permanece libre y se traslada al núcleo, donde interactúa con secuencias específicas de ADN llamadas elementos de respuesta a antioxidantes (ARE), desencadenando la transcripción de genes antioxidantes [43, 44]. Nrf2 puede ser fosforilado en Ser^{40} por varias quinasas sensibles al ROS, facilitando la liberación de Nrf2 del complejo Nrf2-Keap1 y su posterior translocación al núcleo para interactuar con AREs.

La obesidad y la mayoría de las enfermedades crónicas de alta prevalencia, cursan con estrés oxidativo [45, 46]. Tanto la restricción calórica como el ejercicio se asocian a un aumento transitorio de la producción de radicales libres y, en ocasiones, a estrés oxidativo [47-49]. Este aumento de la producción de radicales libres provoca una respuesta contrarreguladora que conduce a un aumento de la capacidad antioxidante y adaptaciones celulares provocadas por los RONS, que mejoran la capacidad y eficacia antioxidante. Se desconoce, no obstante, cual es el mejor modelo de ejercicio para provocar un aumento más eficaz desde la perspectiva cuantitativa y cualitativa de los sistemas antioxidantes en personas como obesidad. Tampoco se sabe cómo interacciona la dieta hipocalórica y el ejercicio en la inducción de los sistemas antioxidantes musculares en seres humanos.

Según el informe Impactur Canarias 2017, realizado por el Gobierno de Canarias y Exceltur, aproximadamente el 40.3% del empleo y el 32.7 % del producto interior bruto de Canarias dependen del turismo (https://www.exceltur.org/wp-content/uploads/2018/07/IMPACTUR-Canarias-2017.pdf). El desempleo en Canarias sigue siendo muy elevado por lo que es necesario fomentar nuevas formas de empleo y actividad económicas, como es el caso del turismo de adelgazamiento. A partir de la experiencia en el diseño de programas de ejercicio para adelgazar proporcionada por el proyecto financiado por el Instituto de Salud Carlos III titulado: “Viabilidad y sostenibilidad del adelgazamiento mediante tratamiento intensificado en pacientes con sobrepeso u obesidad: mecanismos neuroendocrinos y moleculares” (PI14/01509) y el proyecto de investigación financiado por el gobierno de Canarias titulado: “Estudio longitudinal de los efectos de una modificación intensiva del estilo de vida en la composición corporal e indicadores bioquímicos y moleculares de salud en pacientes con

sobrepeso y obesidad: aplicación para la evaluación fisiológica de rutas y sistemas de monitorización del esfuerzo orientados al turismo de salud" (Ref: ProID2017010106), hemos ganado el conocimiento suficiente para generar un modelo de ejercicio factible y atractivo para personas con sobrepeso y obesidad, que al mismo tiempo sirva para fomentar el turismo de salud en Canarias.

Fundamentos biomédicos

Para revertir las alteraciones metabólicas ocasionadas por la obesidad es necesario inducir un déficit energético crónico mediante dieta y/o ejercicio [50-57]. La cirugía bariátrica es una alternativa eficaz para generar un balance energético negativo a través de disminuir la ingestión y absorción de alimentos, que se ha pensado que es más efectiva que la restricción calórica convencional. No obstante, cuatro estudios recientes han demostrado que la forma más popular de cirugía metabólica, llamada cirugía de bypass gástrico Roux-en-Y, no es mejor o puede incluso ser peor que la restricción calórica tradicional para mejorar el control de la glucemia y provocar pérdida de peso [58-61]. Sin embargo, el coste económico y los posibles efectos secundarios de la cirugía bariátrica, hacen de la restricción calórica tradicional combinada con ejercicio una opción mucho más razonable. El problema reside en cómo implementar este tipo de intervenciones para que tengan éxito en la vida real. Otro tipo de intervenciones como la dieta intermitente que limitan el período de ingestión de alimentos a unas pocas horas diarias, no son superiores un año después a lo conseguido con restricción calórica tradicional, mientras que la dieta intermitente se asocia a mayor apetito [62].

En cambio, la eliminación quirúrgica de grasa mediante liposucción de por ejemplo unos 10 kg de grasa subcutánea no mejora la sensibilidad a la insulina [63]. El ejercicio diario permite prevenir el desarrollo de diabetes mellitus tipo 2 incluso en ausencia de pérdida de peso, pero los sujetos que mejoran más su estatus glucémico son los que realizan ejercicio y además consiguen perder peso [64]. Por lo tanto, la pérdida de peso debe ser un objetivo prioritario en el tratamiento de la obesidad y en la prevención de las co-morbilidades. Una característica común de los programas exitosos de adelgazamiento y prevención de la DM2 ha sido el control exhaustivo de la intervención, la creación de grupos de ejercicio controlados y el seguimiento regular de la consecución de los

objetivos de cambio del estilo de vida [55, 56, 65, 66]. Estas características deberían ser contempladas en el diseño de un producto turístico para adelgazar, fomentando estancias repetidas en el tiempo con la finalidad de mantener o incrementar el adelgazamiento conseguido y fidelizar a los "clientes". Entre las estancias en Canarias se podría establecer un asesoramiento telemático en sus países de origen, que podría generar puestos de trabajo adicionales de alto valor añadido y grado de cualificación, que obviamente requerirían un dominio elevado del inglés.

Hasta recientemente, se creía que los efectos beneficiosos del ejercicio se debían únicamente al adelgazamiento inducido por el balance energético negativo. Sin embargo, en los últimos 15 años se ha demostrado que el músculo esquelético sintetiza y libera gran cantidad de hormonas llamadas mioquinas [67], que podrían mediar en los efectos beneficiosos del ejercicio, entre ellos, en la mejora de los sistemas antioxidantes [68-70].

El ejercicio físico regula mejora los sistemas antioxidantes

El ejercicio regular, a través de procesos horméticos desencadenados por ráfagas de RONS generadas durante las contracciones musculares, puede causar un aumento de algunas enzimas antioxidantes [71-73]. Sin embargo, no se sabe si se requiere una mayor capacidad enzimática antioxidante para alcanzar un mayor VO_2max. Al aumentar la capacidad antioxidante de los músculos, el ejercicio regular puede prevenir el estrés oxidativo [74]. El estrés oxidativo puede ser causado por un aumento en la producción de RONS, una reducción en la disponibilidad de antioxidantes o una combinación de ambos [14].

Las personas con una mejor función mitocondrial y una mayor aptitud aeróbica son menos propensas a sufrir los efectos perjudiciales del estrés oxidativo [14]. Por lo tanto, parte de los beneficios del ejercicio para reducir la incidencia de enfermedades crónicas y facilitar un envejecimiento saludable pueden estar relacionados con sus efectos sobre las enzimas antioxidantes y sus factores reguladores. La condición física aeróbica se puede evaluar midiendo el consumo máximo de oxígeno (VO_2max), que proporciona una evaluación integrada del sistema cardiorrespiratorio y la capacidad del músculo para extraer y utilizar el oxígeno. Un alto VO_2max es una característica distintiva de atletas de resistencia de élite, mientras que un VO_2max bajo se asocia con una esperanza de vida

más corta [75] y una mayor mortalidad en poblaciones clínicas [76]. Un VO_2max bajo puede deberse a un suministro reducido de oxígeno, como se observa en pacientes con enfermedades cardiorrespiratorias o a una capacidad reducida para utilizar oxígeno debido a una masa muscular reducida, capilarización o capacidad respiratoria mitocondrial reducida, o su combinación [77, 78]. A pesar de la menor concentración de hemoglobina en sangre en las mujeres, los valores de VO_2max son similares en hombres y mujeres cuando se normalizan según la masa magra de las extremidades inferiores [79]. Esto probablemente se debe a la mayor capacidad de extracción de oxígeno en las mujeres, atribuida a su capacidad respiratoria mitocondrial superior [80]. Algunos complejos y deshidrogenasas respiratorias mitocondriales generan radical superóxido (O_2^-), lo que puede dañar las estructuras y enzimas mitocondriales y dificultar la respiración mitocondrial y la generación de ATP [81]. Esto se previene eficazmente mediante superóxido dismutasas mitocondriales y sarcoplásmicas [14]. Hasta la fecha, la investigación no ha determinado si se requiere un alto nivel de enzimas antioxidantes mitocondriales para lograr VO_2max elevado. Un estudio previo comunicó una correlación positiva entre el VO_2max en $mL.kg^{-1}.min^{-1}$ y las actividades enzimáticas musculares de SOD y catalasa en 12 hombres de entre 17 y 19 años [82]. Estos resultados necesitan confirmación en una muestra de población más amplia, que incluya a hombres y mujeres. Además, ningún estudio ha determinado si los niveles basales de expresión de proteínas Nrf2 y Keap1 en el músculo esquelético humano están vinculados al VO_2max o están más influenciados por otros factores como la edad, la adiposidad o el sexo. Aún se desconoce si existe dimorfismo sexual en la expresión de enzimas antioxidantes en el músculo esquelético humano, como se ha informado para algunos tejidos en roedores [83]. En apoyo, el estradiol estimula la expresión de antioxidantes enzimáticos en algunas células [84, 85], a través de un mecanismo dependiente de Nrf2 [86, 87].

Una investigación reciente mostró por primera vez que el ejercicio exhaustivo reduce la cantidad de proteína Keap1 en el músculo esquelético humano, y la abundancia de Nrf2 aumentó en el agotamiento, lo que sugiere una mayor síntesis *de novo* o una reducción de la degradación proteasomal durante el ejercicio [88]

En la actualidad es difícil conocer la cantidad de Nrf2 o enzimas antioxidantes en el músculo en función del estado físico, la edad o el sexo de los individuos [89-93]. El

estilo de vida sedentario, la obesidad y los problemas musculares relacionados con el envejecimiento se asocian con un aumento del estrés oxidativo [94, 95]. Estudios en humanos han demostrado que los niveles de Nrf2 son más altos en sujetos de edad avanzada que en sujetos con un estilo de vida sedentario [95]. Nrf2 desempeña un papel fundamental en la regulación del metabolismo, la inflamación, la autofagia, la proteostasis, la fisiología mitocondrial y las respuestas inmunológicas [96], es decir, Nrf2 podría ser un factor predictivo de la expectativa de vida.

Debido al envejecimiento, Nrf2 y la subsiguiente respuesta citoprotectora se ven afectados, lo que da lugar a fragilidad y sarcopenia en la vejez junto con un mal rendimiento físico [97-100]. El grado de condición física es crucial para controlar la respuesta de Nrf2, ya que, con el entrenamiento y el ejercicio físico, Nrf2 aumenta, restaurando así la homeostasis redox. Posiblemente po ello, un estilo de vida activo a lo largo de la vida tiene mayores beneficios que comenzar a una edad avanzada [42, 101-103]. Debido a los efectos antioxidantes y antiinflamatorios de la activación de la señalización por Nrf2, el aumento de la expresión de Nrf2 podría contribuir a disminuir la resistencia a la insulina [104]. En pacientes obesos, la actividad de enzimas antioxidantes como SOD está disminuida en comparación con sujetos sanos [105]. Por lo tanto, Nrf2 juega posiblemente un papel crucial en la obesidad y la resistencia a la insulina [106].

III

OBJETIVOS

OBJETIVOS

En el marco de esta tesis doctoral, se han llevado a cabo cuatro estudios independientes, cada uno de ellos con su propios objetivos e hipótesis.

A continuación, presentaremos los objetivos e hipótesis de cada uno de estos estudios:

Estudio 1

- **Objetivo:**

Determinar la magnitud de la "reserva funcional" a través de la realización de series repetidas de ejercicio supramáximo intercaladas con periodos de recuperación bajo condiciones de oclusión de la circulación hasta la incapacidad de reiniciar el ejercicio. Además, investigar los mecanismos moleculares subyacentes que explican la magnitud de la reserva funcional, con especial énfasis en el estudio del papel del eje Nrf2/Keap1 y enzimas antioxidantes críticas en la musculatura esquelética.

- **Hipótesis:**

Hipotetizamos que una mayor proporción de enzimas glucolíticas, como la PFKM, y un mayor porcentaje de MHC II se asociarían con una mayor reserva funcional. Además, a tenor del papel atribuido a las RONS en la fatiga, también hipotetizamos que proteínas involucradas en la homeostasis del calcio, como SERCAs, CSQ1 y CSQ2, junto con enzimas antioxidantes (Catalasa y SODs) podrían desempeñar un papel determinante en la reserva funcional. Además, propugnamos que la reserva funcional podría ser mayor en individuos que experimentan una reducción menor en la oxigenación cerebral durante el ejercicio de alta intensidad.

Estudio 2

- **Objetivo:**

Determinar si niveles más altos de VO_2max requieren una mayor capacidad antioxidante en el músculo esquelético humano, evaluando los niveles de expresión

de proteínas de Nrf2/Keap1 y enzimas antioxidantes como SOD, catalasa y GR en el músculo esquelético humano. Además, investigar si existen diferencias en los niveles de expresión entre sexos.

- **Hipótesis:**

Nuestra hipótesis central es que un mayor VO_2max y el sexo femenino se asociarán con una mayor expresión de Nrf2 y enzimas antioxidantes en el músculo esquelético. Además, esperamos que los niveles de expresión de SOD1 aumenten con la edad, independientemente del VO_2max y el porcentaje de grasa corporal. También hipotetizamos que un índice de masa corporal (IMC) igual o superior a 25 kg/m^2 se asociará con niveles elevados de $pSer^{40}$-Nrf2, $pSer^{40}$-Nrf2/Total Nrf2 ratio y SOD1, después de ajustar diferencias en edad y VO_2max normalizado a la masa magra de las extremidades inferiores.

Estudio 3

- **Objetivo:**

Identificar factores fenotípicos musculares que podrían determinar el rendimiento en sprint en adultos de ambos sexos, centrándose en proteínas y enzimas involucradas en la generación de energía, transporte de oxígeno, regulación redox y manejo del calcio en el músculo esquelético humano.

- **Hipótesis:**

Nuestra hipótesis propugna que los factores musculares que mejoran la regulación redox, como Nrf2/Keap1 y enzimas antioxidantes reguladas por Nrf2, podrían ser determinantes del rendimiento en el sprint. Además, esperamos que una mayor expresión de enzimas implicadas en el manejo del calcio, la generación de energía glucolítica y la utilización de oxígeno se asocie con un aumento en el rendimiento en sprint.

Estudio 4

- **Objetivo:**

Determinar el gasto energético en hombres y mujeres durante el transcurso de un recorrido de caminata prolongada y determinar el gasto energético de la ruta. Además, generar información científica útil para el futuro desarrollo de un producto turístico orientado a la salud, específicamente destinado a turistas que persigan adelgazar y disminuir su estrés oxidativo a través de una estancia en Canarias.

- **Hipótesis:**

En este estudio se hipotetizó que el gasto energético en una ruta de más de 2 horas de duración se incrementaría con la distancia recorrida y que sería similar en hombres y mujeres cuando los valores se corrigen por la masa corporal.

IV

JUSTIFICACIÓN

JUSTIFICACIÓN

La propuesta de esta investigación para una tesis doctoral en la Universidad de Las Palmas de Gran Canaria se justifica en su capacidad para abordar cuestiones de importancia relacionadas con el rendimiento físico y la salud.

Se ha demostrado que la obesidad y muchas enfermedades crónicas de alta prevalencia están vinculadas al estrés oxidativo. Además, tanto la restricción calórica como el ejercicio, dos intervenciones clave en la gestión de la obesidad y las enfermedades crónicas, están asociadas con un aumento temporal en la producción de radicales libres y estrés oxidativo. Sin embargo, este aumento de radicales libres desencadena una respuesta adaptativa en el organismo, que incluye un incremento en la capacidad antioxidante y adaptaciones celulares destinadas a mejorar la eficacia en la neutralización de los RONS.

A pesar de estos avances, todavía existen lagunas en el conocimiento científico. No se sabe cuál es el modelo de ejercicio más efectivo para estimular los sistemas antioxidantes, tanto desde una perspectiva cuantitativa como cualitativa, en personas con obesidad. Además, la interacción entre el ejercicio en la inducción de los sistemas antioxidantes musculares en seres humanos es poco comprendida. Es importante destacar que la mayoría de los estudios anteriores se han centrado en poblaciones masculinas, lo que subraya la necesidad de investigaciones que incluyan a mujeres y aborden las diferencias de género en este contexto.

La justificación se fortalece con la comprensión de que el ejercicio regular tiene un impacto positivo en los sistemas antioxidantes a través de la activación de proteínas reguladoras del sistema antioxidante. La relación Nrf2-Keap1 y las enzimas antioxidantes relacionadas es crucial para el aumento de la capacidad antioxidante y la adaptación celular frente al estrés oxidativo.

Los objetivos generales de la tesis doctoral son: en primer lugar, determinar el rol del sistema antioxidante en la musculatura esquelética en seres humanos, a través de, específicamente 1) estudiar su relación con el fitness cardiorrespiratorio, el envejecimiento y los niveles de grasa corporal y las diferencias entre sexos y 2)

determinar su regulación con diferentes modelos de ejercicio físico. En particular se estudian los niveles de expresión proteica del principal eje regulador del sistema antioxidante (Nrf2/Keap1) y las enzimas antioxidantes musculares. En segundo lugar, contribuir a establecer los fundamentos científicos que permitan optimizar el desarrollo de un producto turístico orientado a la salud, específicamente destinado a turistas que persigan disminuir su estrés oxidativo a través de una estancia en Canarias.

Por lo tanto, esta propuesta de investigación busca llenar un vacío de conocimiento al abordar preguntas importantes acerca de cómo el ejercicio y el estrés oxidativo influyen en la inducción de los sistemas antioxidantes en el músculo esquelético humano. Para ello se han llevado a cabo un estudio exhaustivo de señales celulares clave implicadas en la respuesta antioxidante. La comprensión de estos mecanismos puede tener implicaciones significativas para el tratamiento de la obesidad y las enfermedades crónicas, así como para la propuesta de modelos de ejercicio adecuados para promocionar el turismo de salud en Canarias.

V

CONCLUSIONES

CONCLUSIONES

Estudio 1:

- La reserva funcional tiene una magnitud finita que varía de 0.17 a 2.03 kJ.kg LLM^{-1} en seres humanos jóvenes y sanos, con valores similares en hombres y mujeres.
- La reserva funcional tiene un componente obligatorio que depende de una reserva en capacidad glucolítica (componente glucolítico) y de un componente putativo generado proporcionado por la fosforilación oxidativa (componente aeróbico).
- El componente aeróbico depende de la oxigenación cerebral y de las características fenotípicas de los músculos esqueléticos implicados en el manejo del calcio (expresión de proteínas SERCA1 y 2), el transporte y difusión de oxígeno (mioglobina) y la regulación redox (Keap1).
- El componente glucolítico puede predecirse mediante los niveles de expresión de proteínas de pSer40-Nrf2, el déficit máximo de oxígeno acumulado y los niveles de expresión de proteínas de SOD1.

Estudio 2:

- El nivel de expresión proteína de la proteína SOD2 se asocia al VO_2max en ml.kg LLM^{-1}
- El nivel de expresión proteína de SOD1 aumenta con el envejecimiento.
- Los hombres y las mujeres premenopáusicas tienen niveles similares de expresión de Nrf2 y enzimas antioxidantes en el músculo esquelético humano.
- El sobrepeso y la obesidad se asocian con niveles elevados de expresión de proteínas $pSer^{40}$-Nrf2, la relación $pSer^{40}$-Nrf2/Nrf2 total y SOD1, después de considerar las diferencias en la edad y el VO_2max.

- Un valor más elevado de VO_2max en ml.kg LLM^{-1} se asocia con una mayor expresión basal de ciertas enzimas antioxidantes musculares, lo que podría contribuir a explicar algunos de los beneficios del ejercicio regular.

Estudio 3:

- Las dos variables principales que determinan el rendimiento en sprint en bicicleta ergométrica, son la masa muscular activa y el porcentaje de cadena pesada de la miosina tipo II.

- SDHB y Keap1 también contribuyen a determinar el rendimiento en el sprint, pero en menor medida que las variables anteriores.

Estudio 4:

- El gasto energético al caminar aumenta con la distancia recorrida y es similar para hombres y mujeres.

- El gasto energético al caminar depende sobre todo de la masa corporal.

VI

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VII

APÉNDICE

ESTUDIO 1

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Determinants of the maximal functional reserve during repeated supramaximal exercise by humans: The roles of Nrf2/Keap1, antioxidant proteins, muscle phenotype and oxygenation

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ABSTRACT

When high-intensity exercise is performed until exhaustion a "functional reserve" (FR) or capacity to produce power at the same level or higher than reached at exhaustion exists at task failure, which could be related to reactive oxygen and nitrogen species (RONS)-sensing and counteracting mechanisms. Nonetheless, the magnitude of this FR remains unknown. Repeated bouts of supramaximal exercise at 120% of VO_2max interspaced with 20s recovery periods with full ischaemia were used to determine the maximal FR. Then, we determined which muscle phenotypic features could account for the variability in functional reserve in humans. Exercise performance, cardiorespiratory variables, oxygen deficit, and brain and muscle oxygenation (near-infrared spectroscopy) were measured, and resting muscle biopsies were obtained from 43 young healthy adults (30 males). Males and females had similar aerobic (VO_2max per kg of lower extremities lean mass (LLM): 166.7 ± 17.1 and 166.1 ± 15.6 ml kg $LLM^{-1}.min^{-1}$, P = 0.84) and anaerobic fitness (similar performance in the Wingate test and maximal accumulated oxygen deficit when normalized to LLM). The maximal FR was similar in males and females when normalized to LLM (1.84 ± 0.50 and 2.05 ± 0.59 kJ kg LLM^{-1}, in males and females, respectively, P = 0.218). This FR depends on an obligatory component relying on a reserve in glycolytic capacity and a putative component generated by oxidative phosphorylation. The aerobic component depends on brain oxygenation and phenotypic features of the skeletal muscles implicated in calcium handling (SERCA1 and 2 protein expression), oxygen transport and diffusion (myoglobin) and redox regulation (Keap1). The glycolytic component can be predicted by the protein expression levels of $pSer^{40}$-Nrf2, the maximal accumulated oxygen deficit and the protein expression levels of SOD1. Thus, an increased capacity to modulate the expression of antioxidant proteins involved in RONS handling and calcium homeostasis may be critical for performance during high-intensity exercise in humans.

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Abbreviations

ATP	adenosine triphosphate
ATP5A	subunit of mitochondrial ATP synthase
BSA	bovine serum albumin
Ca^{2+}	calcium ion
COXII	cytochrome *c* oxidase subunit II
CP	constant power
CSQ1	calsequestrin 1
CSQ2	calsequestrin 2
DEXA	dual-energy x-ray absorptiometry
FCR	free circulation recovery
F_IO_2	inspired oxygen fraction
HRmax	maximal heart rate
HRP	horseradish peroxidase
ISR	ischaemic recovery
Keap1	kelch-like ECH-associated protein 1
LLM	lower extremities lean mass
MAOD	maximal accumulated oxygen deficit
MCAv	middle-cerebral artery velocity
MFR	maximal functional reserve
MHC	myosin heavy chain
MHC I	myosin heavy chain type I
MHC IIa	myosin heavy chain type IIa
MHC IIx	myosin heavy chain type IIx
NDUFB8	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8
NF-κB p65	nuclear factor NF-kappa-B p65 subunit
NIRS	near-infrared spectroscopy
Nrf2	nuclear factor (erythroid-derived 2)-like 2
NOX	NADPH oxidase
P38 MAPK	p38 mitogen-activated protein kinase
PCr	phosphocreatine
PFKM	phosphofructokinase
P_IO_2	partial pressure of inspired O_2
PVDF	polyvinylidene fluoride
RER	respiratory exchange ratio
RONS	reactive oxygen and nitrogen species
ROS	reactive oxygen species
Rpm	revolutions per minute
SDHB	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8
SERCA	sarco-endoplasmic reticulum Ca^{2+} ATPase
SODs	superoxide dismutase
TOI	tissue oxygenation index
TOI_{MIN}	minimal 1-s rolling average TOI value during ischaemia
TOI_{OBV}	mean TOI value registered during exercise
UQCRC2	ubiquinol-cytochrome-c reductase complex core protein 2
V_E	ventilation
VO_2	oxygen uptake
VO_2max	maximal oxygen uptake
W	watts
Wmax	peak power output during the incremental exercise test to exhaustion
Wmean	mean power output during the Wingate test
Wpeak1-s	peak power output in 1-s averages during the Wingate test
Wpeak	instantaneous peak power output during the Wingate test
XO	xanthine oxidase

1. Introduction

During high-intensity exercise reactive oxygen and nitrogen species (RONS) production is exacerbated due to high oxidative and substrate level phosphorylation rates, and acidification caused by high glycolytic rates [1–4]. While RONS have been implicated in muscle fatigue [1,3–5], exercise training reduces RONS production and enhances the antioxidant capacity of skeletal muscle by mechanisms mediated by the nuclear factor erythroid-derived 2-like 2 (Nrf2) [6–8]. Nrf2 activity depends on the Kelch-like ECH-associated protein 1 (Keap1) levels, which under unstressed conditions, acts as an adaptor for a ubiquitin E3 ligase complex, which tags Nrf2 for proteasomal degradation [9,10]. Keap1 is a redox sensor protein that, upon oxidative/electrophilic stress, stabilizes the Keap1-Nrf2 complex, impeding Nrf2 degradation [10]. When the level of free Keap1 is low, newly synthesized Nrf2 can translocate to the nucleus, where it interacts with antioxidant response elements (AREs), DNA sequences present in genes not only involved in the antioxidant response, but also in mitochondrial biogenesis, and metabolism, as well as other functions [9,10]. Interestingly, disruption of Keap1 in mice has been associated with enhanced exercise endurance [11].

Nevertheless, most studies indicate that acute oral administration of antioxidants to humans does not improve high-intensity exercise performance [12,13]. This contrasts with animal studies and *in vitro* experiments showing that antioxidants counteract fatigue with a dose-response curve that is "U" shaped, i.e., an excessive amount of antioxidants appears to be detrimental [14]. The apparent insensitivity of human skeletal muscle to antioxidant administration may depend on the dose and type of antioxidant, the characteristics of the subjects and type of exercise employed. Thus, a novel exercise paradigm using repeated cycles of exercise to exhaustion followed immediately by a brief occlusion of the circulation and resumption of exercise with open circulation to elicit repeated cycles of ischaemia-reperfusion has been employed to investigate mechanisms of functional reserve [15–19]. This protocol can be used to test whether the characteristics of the subjects and their muscle phenotype, including resting expression of Keap1 and Nrf2, could explain differences in exercise performance and metabolism in humans repeatedly exercising to exhaustion.

During whole-body exercise at supramaximal exercise intensity, i.e., above the intensity eliciting VO_2max, part of the energy required to supply the ATP demand must be provided by substrate level phosphorylation leading to accumulation of metabolites, fatigue, and task failure [20,21]. Nevertheless, part of the exercise capacity can be recovered after a few seconds or minutes of rest allowing the repetition of several supramaximal exercise bouts, although the work that can be produced in successive repetitions is progressively reduced [22,23]. This quick partial recovery of exercise capacity after task failure has been explained by a rapid resynthesis of phosphocreatine (PCr) [24–27] and proton efflux allowing intracellular pH recovery [25,28–30], although intracellular pH recovery requires several minutes [29]. PCr resynthesis depends on O_2 availability [29,31] and no PCr recovery is observed if ischaemia is applied at exhaustion [15,32] or if exercise is performed during ischaemia and the occlusion remains in place until exhaustion [33]. However, we have shown that a partial recovery of exercise capacity is possible despite the application of ischaemia at the end of high-intensity exercise to exhaustion [15–19]. Thus, some "functional reserve" or capacity to produce power at the same level or higher than reached at exhaustion exists at task failure, which depends on the glycolytic component of substrate level phosphorylation [15,16].

In previous studies, this "functional reserve" was observed after a single exercise bout to exhaustion [15], as well as after two bouts of exercise at 120% of VO_2max [16]. This "functional reserve" should have a finite magnitude. However, in preceding experiments it was not possible to determine its actual value since the thirty-six subjects tested were able to show some functional reserve after two bouts of supramaximal exercise at 120% of VO_2max [16]. It remains unknown how

many bouts could be performed, and which factors may determine the magnitude of the "functional reserve".

Therefore, the primary aim of this investigation was to determine the magnitude of the "functional reserve" using repeated bouts of supramaximal exercise interspaced with 20s recovery periods with occlusion of the circulation until incapability to re-start exercising. Additionally, we aimed to determine what mechanisms could explain the magnitude of the functional reserve. We hypothesized that a higher proportion of glycolytic enzymes, such as phosphofructokinase (PFKM), and a higher percentage of myosin heavy chain type II (MHC II) would associate with a higher functional reserve. Fatigue and task failure during high-intensity exercise has been also attributed to the effect of RONS, which may inactivate critical enzymes and interfere with calcium transients and calcium reuptake [1–4,34]. Therefore, we hypothesized that some proteins involved in calcium homeostasis like the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCAs) and calsequestrin 1 and 2 (CSQ1 and CSQ2, respectively), as well as the more abundant antioxidant enzymes in skeletal muscle (i.e., catalase and SODs) could play a role as determinants of the "functional reserve".

Insufficient muscle and brain oxygenation can contribute to fatigue, and particularly a lower brain oxygenation during exercise performed until exhaustion has been associated with fatigue in several studies [35–38]. Thus, we also hypothesized that the "functional reserve" may be greater in the subjects experiencing a lesser reduction in brain oxygenation during high-intensity exercise.

Thus, to achieve these aims we have conceived a new exercise protocol which allows for testing the response of skeletal muscle to repeated episodes of ischaemia-reperfusion under conditions of extreme fatigue in humans.

2. Materials and methods

2.1. Subjects

Thirty males and 13 females, all healthy and physically active agreed to participate in this investigation (Table 1). The inclusion criteria for participation in this investigation were: aged 18–35 years old; non-smoking; normal resting electrocardiogram, no chronic diseases or recent surgery; body mass index between 18 and 30 $kg{\cdot}m^{-2}$; no medical contraindications to exercise and no history of disease requiring medical treatments lasting longer than 15 days during the preceding six months. Subjects were requested to avoid strenuous exercise 48 h before the laboratory test and not to drink carbonated, caffeinated and alcohol-containing beverages during the 24 h preceding all tests. During the study period, subjects were also requested to abstain from the consumption of drugs, medications, and any dietary supplements. Sex and gender of the participants were defined based on self-report during participant recruitment. All participants reported *cis*-gender, and thereafter the terms males and females were applied in the study analysis and reporting [39]. All females were eumenorrheic, without taking oral contraceptives and were evaluated randomly in different phases of the menstrual cycle [40]. This approach is based on the similar sprint and high-intensity exercise responses observed in different phases of the menstrual cycle [41–43].

The study was approved by the Ethical Committee of the University of Las Palmas de Gran Canaria (Ref.: CEIH-2017-13) and performed in accordance with the standards set by the latest revision of the Declaration of Helsinki, except for registration in a database. All subjects signed a written informed consent before the start of the study.

2.2. Study overview

The study included the following phases: a) familiarization and pre-testing; b) assessment of VO_2max and the relationship between VO_2 and exercise intensity; c) Wingate test, d) supramaximal exercise tests to determine the functional reserve at exhaustion; and e) obtaining muscle biopsies (Fig. 1).

Table 1
Physical characteristics and ergoespirometric variables (mean ± SD).

	Males (n = 30)	Females (n = 13)	*p*
Age (years)	22.5 ± 2.1	22.2 ± 1.5	0.624
Height (cm)	176.7 ± 7.6	162.2 ± 4.5	**0.000**
Weight (kg)	73.3 ± 7.5	58.5 ± 11.6	**0.000**
% body fat	19.3 ± 5.1	27.9 ± 4.2	**0.000**
Legs lean mass (kg)	20.2 ± 1.7	13.9 ± 2.3	**0.000**
HRmax ($Beats{\cdot}min^{-1}$)	193.2 ± 8.3	195.1 ± 7.9	0.475
VO_2max ($mL{\cdot}kg^{-1}{\cdot}min^{-1}$)	46.3 ± 6.4	39.9 ± 3.5	0.002
VO_2max ($mL{\cdot}kg\ LLM^{-1}{\cdot}min^{-1}$)	166.7 ± 17.1	166.1 ± 15.6	0.913
Wmax (W)	263.2 ± 36.0	188.7 ± 40.0	**0.000**
MHC I	34.0 ± 12.4	53.5 ± 11.4	**0.000**
MHC IIa	51.2 ± 9.2	36.3 ± 6.5	**0.000**
MHC IIx	14.9 ± 9.6	10.2 ± 6.3	0.120
MHC I + IIa	85.2 ± 9.7	89.8 ± 6.3	0.123
120% VO_2max CI (first bout, best performance)			
Time to exhaustion (s)	117.6 ± 39.2	131.9 ± 40.3	0.280
Power (W)	322.8 ± 49.3	219.4 ± 42.6	**0.000**
Power ($W{\cdot}kg\ LLM^{-1}$)	16.0 ± 2.2	15.7 ± 1.4	0.691
Work ($kJ{\cdot}kg\ LLM^{-1}$)	1.84 ± 0.50	2.05 ± 0.59	0.218
VO_2 ($mL{\cdot}kg\ LLM^{-1}{\cdot}min^{-1}$)	129.1 ± 16.0	132.1 ± 13.3	0.561
O_2/Work ($mL{\cdot}kJ$- [1])	133.5 ± 19.5	140.1 ± 18.2	0.304
O_2 demand (mL)	7806 ± 2867	6012 ± 1803	**0.044**
Accumulated O_2 (mL)	5113 ± 2225	3986 ± 1369	0.099
O_2 deficit (mL)	2692 ± 937	2026 ± 689	**0.026**
O_2 deficit ($mL{\cdot}kg^{-1}$ BW)	36.6 ± 11.4	35.1 ± 11.6	0.690
O_2 deficit ($mL{\cdot}kg\ LLM^{-1}$)	132.4 ± 40.7	145.5 ± 42.3	0.343
% Anaerobic Energy	35.4 ± 6.9	34.2 ± 7.8	0.604
RER	1.18 ± 0.11	1.08 ± 0.11	**0.009**
V_E ($L{\cdot}min^{-1}$)	89.5 ± 17.1	68.4 ± 12.6	**0.000**
RR ($Breaths{\cdot}min^{-1}$)	40.0 ± 6.6	41.7 ± 6.7	0.442
V_E/ VO_2	33.5 ± 5.5	34.6 ± 5.1	0.547
V_E/ VCO_2	29.8 ± 3.7	32.8 ± 5.4	**0.039**
$P_{ET}O_2$ (mmHg)	110.2 ± 4.7	111.8 ± 5.0	0.322
$P_{ET}CO_2$ (mmHg)	39.6 ± 4.5	34.2 ± 5.2	**0.001**
Vastus Lateralis TOI (a.u.)	62.2 ± 17.4	66.6 ± 4.9	0.381
Frontal lobe TOI (a.u.)	68.5 ± 5.5	63.4 ± 7.1	**0.016**
MCAv ($mm{\cdot}s^{-1}$)	44.7 ± 26.6	30.5 ± 35.1	0.153

HRmax: maximal heart rate; VO_{2max}: maximal oxygen uptake; Wmax: maximal intensity during the incremental exercise test to exhaustion; LLM: lean mass of the lower extremities; Accumulated O_2: total amount of O_2 consumed during the test; and the average values during the 120% CP test for: RER, respiratory exchange ratio; V_E, pulmonary ventilation; RR, respiratory rate; $P_{ET}O_2$, end tidal O_2 pressure; $P_{ET}CO_2$, end tidal CO_2 pressure; TOI, tissue oxygenation index; MCAv, middle cerebral artery velocity. Note: the supramaximal test data correspond to the session with the best performance time. P values based on two-tailed unpaired t-tests.

2.3. Familiarization and pre-testing

Volunteers reported to the laboratory after a 12-h overnight fast for the assessment of their body composition by dual-energy x-ray absorptiometry (Lunar iDXA, GE Healthcare, Milwaukee, WI, USA) [44]. On a different visit, subjects were familiarized with the experimental procedures which included an incremental exercise to exhaustion with verification, sprint exercise (30-s Wingate all-out test) and post-exercise occlusion of the circulation. After that, subjects reported to the laboratory to complete different experimental tests on separate days.

2.4. Assessment of VO_2max and the VO_2/intensity relationship

The VO_2max, maximal heart rate (HRmax), and maximal power output (Wmax) were determined in normoxia (F_IO_2: 0.21, P_IO_2: 144 mmHg) with an incremental exercise test until exhaustion with verification [45]. For this test, subjects reported to the laboratory at least 4 h after the last ingestion of food. The test started with 3 min at 20 W, followed by 15 W and 20 W increases every 3 min in females and males, respectively, until the respiratory exchange ratio (RER) was ≥1.00. After

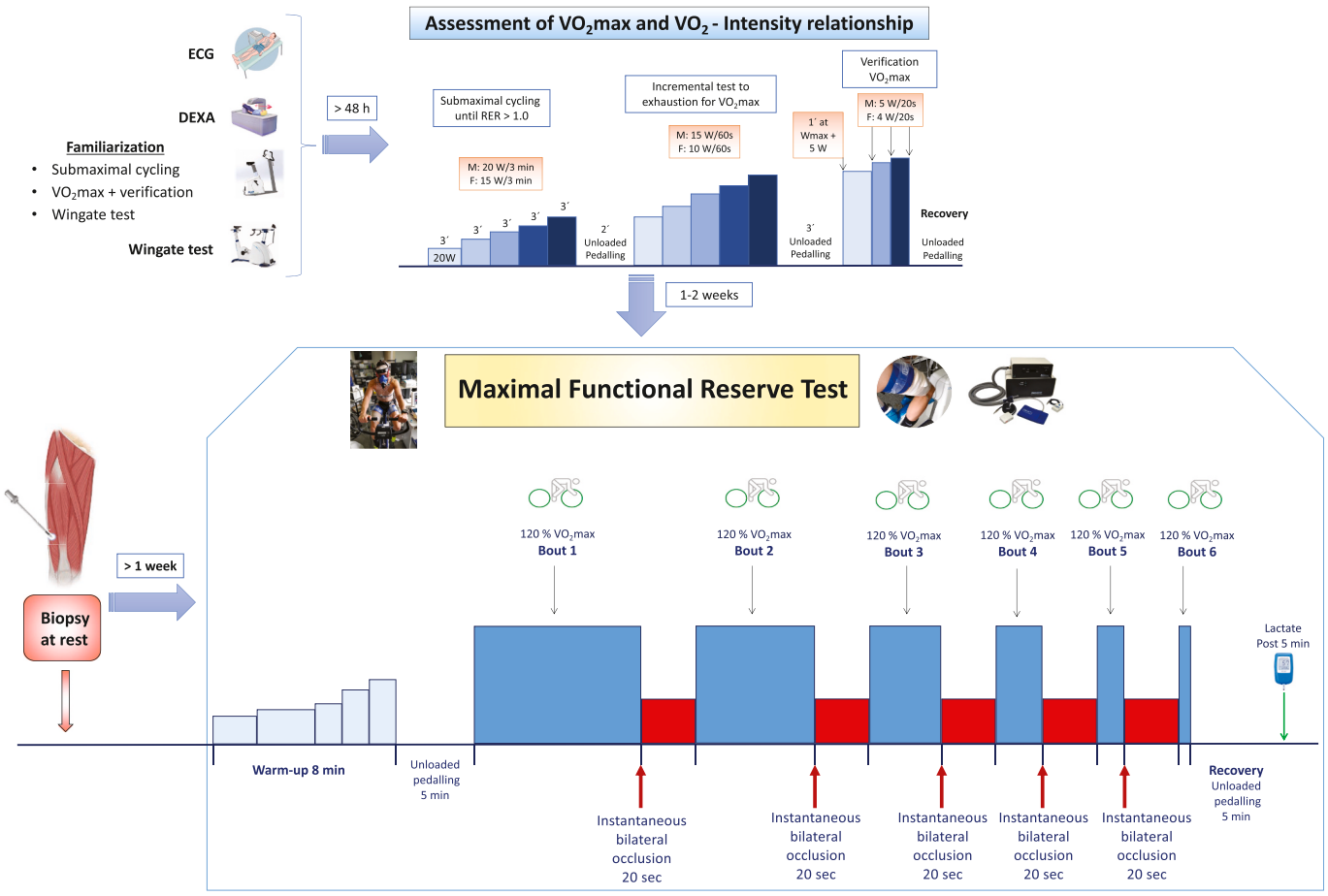

Fig. 1. Schematic representation of the study phases and exercise protocols.
Forty-three physically active participants (30 males and 13 females) were recruited for the study. After fulfilling inclusion criteria, a DEXA scan was performed after a 12-h overnight fast. On another visit, participants performed a familiarization session with the cycle ergometer exercise protocols that included submaximal cycling exercise, an incremental test until exhaustion with verification, a 30-s Wingate test and post-exercise occlusion of the circulation. On a third visit, participants carried out a test to assess VO_2max and the VO_2/intensity relationship. At least one week apart, the subjects were submitted to a resting muscle biopsy, which was separated by a minimum of one week from the performance of the maximal functional reserve tests (MFR). The MFR sessions consisted in two experimental sessions. In each of them, after a standardized warm-up, subjects performed six bouts of supramaximal constant intensity exercise at 120% of VO_2max until exhaustion, interspersed either with 20 s of recovery periods with application of immediate post-exercise ischaemia at exhaustion (ischaemic recovery session) or with 20 s of recovery with free circulation (free circulation recovery session), in random order. At the start of the 2nd to 6th bouts in the ischaemic recovery session, the cuffs were deflated instantaneously, to allow for restoration of the circulation during the subsequent bout. The cuffs located around the two thighs were instantaneously inflated at 300 mmHg during the sessions with ischaemic recovery to elicit total occlusion of the circulation of both lower extremities and impede metabolic recovery. The schematic presented for the MFR test corresponds to the ischemic session. In 13 volunteers a muscle biopsy was obtained before and immediately after the ISR test.

that, the load was increased by 10 W and 15 W every minute, in females and males, respectively, until exhaustion. The highest intensity attained in the test was taken as the Wmax of the incremental exercise. At exhaustion, the ergometer was unloaded, and slow pedalling (30–40 rpm) continued for 3 min. At the third minute of active recovery, the verification test was initiated at Wmax +5 W during 1 min, followed by a 4 and 5 W increase (females and males, respectively) every 20 s until exhaustion.

Oxygen uptake (VO_2) was assessed by indirect calorimetry with a metabolic cart (Vyntus, Jaeger-CareFusion, Höchberg, Germany) operated in breath-by-breath mode. The gas analysers were calibrated immediately before each test using room air (20.93% O_2 and 0.05% CO_2) and high-grade certified gases provided by the manufacturer containing 16% O_2 and 5% CO_2. The volume flow sensor was calibrated at low (0.2 L/s) and high (2 L/s) rates immediately before each test. The validity of this metabolic cart was established by a butane combustion test [46], and its reliability checked during submaximal and maximal exercise intensities [47]. Respiratory variables were analysed breath-by-breath and averaged every 20 s during the incremental exercise tests. The highest 20-s averaged VO_2 recorded during either the incremental test including the verification phase or the supramaximal exercise tests was taken as the VO_2max [47]. Heart rate (HR) was recorded continuously with a sampling frequency of 1 s during all exercise tests via short-range radiotelemetry (RS400 and RS800, Polar Electro, Woodbury, NY, USA).

2.5. Wingate test

On a different day subjects reported to the laboratory to perform a 30-s Wingate test on the cycle ergometer set on isokinetic mode at 80 rpm (Lode Excalibur, Groningen, The Netherlands). Prior to the Wingate test a standardized warm-up was performed with 1 min of unloaded pedalling, followed by 2 min at 40 W or 60 W, 3 min at 60 or 80 W, 1 min at 80 or 100 W, 1 min at 100 or 120 W and 1 min at 120 or 140 W for females and males, respectively. This was followed by 5 min of unloaded pedalling at low cadence (20–40 rpm) and then the Wingate test was started. The data were analysed to obtain instantaneous peak power output (Wpeak), the peak power output in 1-s averages (Wpeak1-s) and the mean power output (Wmean).

2.6. Assessment of the functional reserve at exhaustion (maximal functional reserve tests)

The functional reserve at exhaustion was determined 1–2 weeks after the assessment of VO_2max as previously reported [16]. From the total number of volunteers (n = 43), twenty-four males and six females reported to the laboratory on two occasions, one or two weeks apart, hereafter called ischaemic (ISR) and free circulation (FCR) recovery sessions (Fig. 1), while another group of 6 males and 7 females performed only the ISR protocol. The ISR session was utilized to determine the MFR, while the FCR was used as a control. The tests were performed approximately at the same time in both conditions, and subjects were requested to record the last meal ingested and reproduce it thoroughly before both experimental sessions. For those tests performed in the morning, subjects were requested to ingest a light breakfast, which should have been ended at least 1 h prior to the scheduled time for arrival to the laboratory. For those tests performed in the afternoon, only light meals ingested at least 4 h before the test were permitted. One hour after their arrival to the laboratory, the protocol described in Fig. 1 as "Maximal Functional Reserve Test" (MFR) started.

The ISR and FCR sessions were planned to include six bouts of constant-power exercise to exhaustion interspaced by 20 s recovery periods with (ISR) or without (FCR) application of total occlusion of the circulation to the lower extremities. The intensity of the supramaximal exercise bouts was set at 120% of VO_2max, to facilitate the attainment of the maximal accumulated oxygen deficit during the first bout of constant-power exercise on the cycle ergometer [48,49]. For all tests, volunteers were instructed to maintain the pedalling rate steady at 80 rpm (±3 rpm) and remain seated on the cycle ergometer, i.e., no standing pedalling was permitted. The ergometer seat and handlebar configuration were adjusted for comfort during the first visit and replicated in subsequent sessions. In all instances, exhaustion was defined by the incapacity to maintain a pedalling cadence above 50 rpm during 5 s or by the sudden stop of pedalling. Strong verbal encouragement was provided for the continuation of the exercise, particularly if pedalling rate was declining or task failure imminent.

In the ISR session, the circulation of both lower extremities was instantaneously occluded at exhaustion after each bout. Right at the restart of the second and successive bouts, the cuffs were instantaneously released and the circulation fully re-established. If a given subject was not able to start exercising at 120% VO_2max, the occlusion was instantaneously re-instated for another 20 s, while power was set at 100% of VO_2max. Thereafter the bouts were repeated at 100% of VO_2max until reaching the sixth bout or until incapacity to re-start pedalling. For those subjects performing only the ISR protocol, the sixth bout was followed by an additional occlusion of the circulation of both legs and a muscle biopsy was taken ~10s after exhaustion with the occlusion in place.

The same approach was followed during the FCR sessions, but with the subjects performing unloaded pedalling (~20 rpm) during the 20 s recovery periods, to minimize the risk of orthostatic post-exercise hypotension [50]. In the 20 s recovery period, after 15 s the subjects were given a 5 s reverse countdown and prompted to restart pedalling as fast as possible until the pedal cadence surpassed 80 rpm, which was the target pedalling rate for all bouts. After the last bout of the ISR and FCR tests, the subjects rested in the supine position and in 5 min a 5 μL blood sample was obtained from the hyperaemized earlobe to measure the capillary blood lactate concentration (Lactate Pro 2, Arkray Inc., Kyoto, Japan).

Before the exercise, while the subjects were resting supine, bilateral 10 cm wide cuffs were placed around the thighs, as close as possible to the inguinal crease, and connected to a rapid cuff inflator (SCD10, Hokanson E20 AG101, Bellevue, WA, USA) as previously reported [15,51]. Each session started with a warm-up consisting of 1 min of unloaded pedalling, followed by 2 min at 60 or 40 W, 3 min at 80 or 60 W, 1 min at 100 or 80 W, 1 min at 120 or 100 W and 1 min at 140 or 120 W for males and females, respectively. This was followed by 5 min of unloaded pedalling at low cadence (20–40 rpm). Then the subjects stopped pedalling, and the ergometer was set in hyperbolic mode at the load corresponding to their 120% of VO_2max to perform the MFR test.

Cerebral and muscular oxygenation were assessed using near-infrared spectroscopy (NIRS, NIRO-200NX, Hamamatsu Photonics, Japan) employing spatially-resolved spectroscopy to obtain the tissue oxygenation index (TOI) using a path-length factor of 5.92 [52]. One NIRS optode was placed on the right frontoparietal region at 3 cm from the midline and 2–3 cm above the supraorbital crest, to avoid the sagittal and frontal sinus areas [50]. A second optode was placed in the lateral aspect of the thigh at middle length between the patella and the anterosuperior iliac crest, over the middle portion of the m. *vastus lateralis*. The *vastus lateralis* fractional extraction index (TOI O_2 extraction index) was obtained as TOI_{OBV} - TOI_{MIN}, where TOI_{OBV} is the mean TOI value registered during exercise and TOI_{MIN} is the minimal 1-s rolling average TOI value registered during ischaemia, as previously reported [16]. The validity of the TOI O_2 extraction index has been shown by direct measurement of O_2 extraction by measuring arterial and venous femoral O_2 content during exercise in males and females [16]. The mean blood flow velocity in the middle-cerebral artery velocity (MCAv) was measured as an estimate of cerebral blood flow using two Doppler 2 MHz transducers applied bilaterally over the middle transtemporal window, as previously described [50].

The experiments were performed in an air-conditioned laboratory with an ambient temperature of ~21 °C, a relative humidity of 60–80%, and ~735 mmHg atmospheric pressure. All exercise tests were carried out on the same cycle ergometer (Lode Corival, Lode BV, Groningen, The Netherlands), which maintains the exercise intensity constant despite variations in pedalling rate.

The O_2 demand during the supramaximal exercise bouts was estimated from the linear relationship between the last minute averaged VO_2 of each load, from 20 to 40 W to the highest intensity with an RER <1.00. The accumulated oxygen deficit (AOD, an estimate of the energy provided by substrate-level phosphorylation), representing the difference between O_2 demand and accumulated O_2, was determined as previously reported [15,16,49]. The AOD in the first best bout was taken as the maximal accumulated oxygen deficit (MAOD) [53,54]. The contribution of anaerobic energy metabolism to the total energy yield was calculated as MAOD x 100/O_2 demand. Since during the occlusions the myoglobin O_2 stores are depleted and PCr is not resynthesized [15,32,55,56], the totality of the O_2 deficit measured during the second and subsequent bouts in the ISR sessions corresponded to the energy supplied by the glycolytic component of substrate-level phosphorylation. The MFR was calculated as the sum of O_2 deficits incurred from the second to the sixth bout, or the last exercise bout performed at 100% of VO_2max. The total O_2 deficit was calculated by adding the MFR to the MAOD.

2.7. Muscle biopsies

For biopsy sampling, volunteers reported to the laboratory at 07.00 h, following a 12-h overnight fast and a resting muscle biopsy was obtained from the middle portion of the m. *vastus lateralis* of one of the two thighs, assigned randomly, using the Bergstrom's technique with suction, as previously reported [15]. From the 13 participants (6 males and 7 females) performing only the ISR session, signalling responses Pre and Post-MFR tests were assessed in 12, due to one post-MFR missing value in one male participant. For this purpose, one biopsy was performed approximately ~10 min before initiating the MFR test and immediately after the sixth bout from the same leg during ischaemia. The needle was directed distally with a 45° inclination for the Pre-MFR biopsy, and the skin incision was covered with a provisional plaster that allows a rapid removal at exhaustion for fast collection of the Post MFR biopsy. All biopsies were immediately frozen in liquid nitrogen and stored at – 80 °C until analysed.

2.8. Protein extraction and western blotting

Whole skeletal muscle lysates were prepared as previously reported [57], and total protein concentration quantified using the bicinchoninic acid assay [58]. Briefly, approximately 10 mg of muscle were homogenized in urea lysis buffer (6 M urea, 1% SDS), 50X Complete protease inhibitor and 10X PhosStop phosphatase inhibitor cocktails (Roche). Subsequently, the lysate was centrifuged for 12 min at 25,200 g at 16 °C. The resulting supernatant containing the protein fraction was diluted with electrophoresis loading buffer (160 mM Tris-HCl, pH 6.8, 5.9% SDS, 25.5% glycerol, 15% β-mercaptoethanol- bromophenol blue). The optimal amount of total protein from experimental samples to be loaded and the antibody concentrations for each assay were first determined by loading a gradient of control protein extracts (non-interventional human muscle prepared similarly as the experimental samples) in different amounts ranging from 1 to 35 μg. After confirming linearity within this range, equal amounts of protein of each sample (1.5–15 μg) were loaded and electrophoresed with SDS-PAGE using the system of Laemmli [59] and proteins were transferred onto the polyvinylidene fluoride (PVDF) membranes for protein blotting (Bio-Rad Laboratories, Hercules, CA, USA). To compensate for variability between gels, the samples from each subject were run onto the same gel together with an equal protein amount from an internal control (same as during linearity optimization) loaded in triplicate or quadruplicate. The densitometric value of the protein of interest was normalized to the mean value of the control sample.

Membranes were blocked for 1 h in in either 4% bovine serum albumin or 2.5–5% non-fat dried milk powder (blotting-grade blocker) diluted in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) (BSA or Blotto blocking buffer) and incubated overnight at 4 °C with primary antibodies. Antibodies were diluted in 4% BSA-blocking buffer or 2.5–5% Blotto-blocking buffer. After incubation with primary antibodies, the membranes were washed and incubated for 1 h at room temperature with an HRP-conjugated anti-rabbit or anti-mouse antibody (diluted 1:5000 to 1:20000 in 5% Blotto blocking buffer in all instances) and subsequent chemiluminescent visualization with Clarity™ Western ECL Substrate (Bio-Rad Laboratories) using the ChemiDocTM Touch Imaging System (Bio-Rad Laboratories). Densitometry band quantification was performed with the Image Lab© software 5.2.1 (Bio-Rad Laboratories). Equal loading and transfer efficiency was verified by staining the membranes with Reactive Brown 10 (Sigma-Aldrich, St. Louis, MO, USA). The corresponding catalogue numbers of primary antibodies were as follows: $pSer^{40}$ Nrf2 (no. ab76026), Nrf2 (no. ab62352), SOD1 (no. ab16831) and the anti-OXPHOS premixed cocktail antibody (total OXPHOS human antibody cocktail, no. ab110411) purchased from Abcam (Cambridge, UK); catalase (no. 14097), SOD2 (no. 13141) and myoglobin (D2F5X, Rabbit mAb #25919), $pThr^{180}/Tyr^{182}$ p38 MAPK (no. CS9211), p38 MAPK (no. 9212), and $pSer^{536}$ NFκB p65 (no. 3033), NFκB p65 (no. 3034) from Cell Signalling Technology (Danvers, MA, USA); calsequestrin1 (no. C0618), calsequestrin2, (no. 3868), SERCA1 (no. WH0000487M1) and SERCA2 (no. S1439) from Sigma-Aldrich and Keap1 (no. 10503-2-AP), phosphofructokinase1 (PFKM) (55028-1-AP) and citrate synthase (16131-1-AP) were purchased from Proteintech (Rosemont, IL, USA). The secondary HRP-conjugated goat anti-rabbit (no. 111-035-144) and the HRP-conjugated goat anti-mouse (no.115-035-003) antibodies were acquired from Jackson ImmunoResearch Inc. (West Grove, PA, USA). A more detailed description of materials and procedures used for western blotting is available in the Supplementary Table 1.

2.9. Myosin heavy chain analysis

Determination of MHC isoform proportions was performed on the muscle biopsies using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Experimental samples from the exact same aliquots as used for western blotting were loaded (7.5–10 μg) in triplicate onto the same gel, together with two internal control samples. The inclusion of two lanes loaded with the same internal control in all gels was used for quality check and accurate quantification of the variability of the assays, and not for normalization purposes. Experimental samples and controls were run at 4 °C on an SDS-PAGE gel containing a 3% acrylamide (v/v) phase (stacking gel) for ~12 h at 70 V and afterwards on a 6% acrylamide (v/v) and 30% glycerol (v/v) phase (resolving gel) for ~20 h at 350 V. Subsequently, the gels were Coomassie stained for ~1 h followed by distaining with a 40% methanol (v/v) and 10% glacial acetic acid (v/v) solution for ~ 1 h and lastly by background subtraction submerging the gel in distilled water for ~1 h. Then, the MHC isoform content was determined by scanning the gel with a densitometry scanner (GS-800 Imaging Densitometer, Bio-Rad Laboratories, Hercules, CA, USA) and quantified with the Image Lab© software 5.2.1 (Bio-Rad Laboratories).

2.10. Statistics

The Gaussian distribution of variables was determined with the Shapiro-Wilks test, and when required, data were transformed logarithmically before further analysis. Muscle protein expression values in biopsies taken before and after exercise were compared using a paired *t*-test. Differences between males and females were determined using an unpaired *t*-test. Linear relationships between variables were examined by simple and multiple linear regression analyses. Unless otherwise stated, results are reported as the mean ± standard deviation (SD). Statistical significance was set at $p < 0.05$. Statistical analyses were performed using IBM SPSS Statistics v.21 for Mac (SPSS Inc., Chicago, IL, USA) and Jamovi v1.8.1. (Jamovi project, 2021).

3. Results

3.1. General physical characteristics and performance

The physical characteristics and performance during the incremental exercise to exhaustion and the best 120% constant-power first bout (120% CP) are reported in Table 1 for males and females. The performance achieved during the Wingate test is reported in Table 2. Males and females had comparable VO_2max per kg of LLM (166.7 ± 17.1 and 166.1 ± 15.6 ml·kg LLM^{-1}.min^{-1}, P = 0.91) and had similar peak and mean power output per kg of LLM in the Wingate test (Table 2), as well as MAOD values normalized to LLM during the ISR test (Table 1). VO_2max per kg of LLM was associated with the expression levels of the mitochondrial proteins COXII ($r = 0.52$, $P < 0.001$, $n = 43$), UQCRC2 ($r = 0.33$, $P = 0.029$, $n = 43$), ATP5A ($r = 0.52$, $P < 0.001$, $n = 43$), and NDUFB8 ($r = 0.38$, $P = 0.019$, $n = 43$). VO_2max per kg of LLM was also associated with MHCI + IIa ($r = 0.50$, $P < 0.001$, $n = 43$) and SOD2

Table 2
Wingate test performance (mean ± SD).

	Males (n = 29)	Females (n = 11)	*p*
Wpeaki (W)	1010 ± 206	707 ± 185	**0.000**
Wpeak1-s (W)	812 ± 149	564 ± 140	**0.000**
Wmean (W)	575 ± 95	381 ± 91	**0.000**
Wpeaki (W·kg BW^{-1})	13.8 ± 2.6	12.7 ± 3.0	0.245
Wpeak1-s (W·kg BW^{-1})	11.1 ± 2.0	10.1 ± 2.3	0.173
Wmean (W·kg BW^{-1})	7.9 ± 1.0	6.8 ± 1.5	**0.019**
Wpeaki (W·kg LM^{-1})	18.1 ± 3.3	18.2 ± 3.9	0.881
Wpeak1-s (W·kg LM^{-1})	11.1 ± 2.0	10.1 ± 2.3	0.173
Wmean (W·kg LM^{-1})	10.3 ± 1.3	9.9 ± 1.9	0.432
Wpeaki (W·kg LLM^{-1})	50.0 ± 9.2	51.8 ± 11.0	0.595
Wpeak1-s (W·kg LLM^{-1})	40.3 ± 6.6	41.5 ± 8.5	0.634
Wmean (W·kg LLM^{-1})	28.5 ± 3.5	28.1 ± 5.7	0.796

Wpeaki: instantaneous peak power output; Wpeak1-s: peak power output for 1-s averages; Wmean: mean power output. BW: body weight; LM: whole body lean mass; LLM: lean mass of the lower extremities. P values based on two-tailed unpaired t-tests. One male and one female did not perform the Wingate test.

protein expression levels (r = 0.42, P = 0.006, n = 42).

3.2. Impact of the exercise protocol on p38 MAPK and NF-κB p65 phosphorylation as surrogate indicators of RONS-induced signalling

The ISR protocol substantially increased the phosphorylation of $pThr^{180}/Tyr^{182}$ p38 MAPK (P = 0.016, n = 12). Additionally, the ratio of phosphorylated $pThr^{180}/Tyr^{182}$ p38 MAPK to total p38 MAPK exhibited a notable elevation (P = 0.051, n = 12). The latter was accompanied by increased phosphorylation of $pSer^{536}$ NF-κB p65 (P = 0.004, n = 12), along with an elevated ratio $pSer^{536}$ NF-κB p65/NF-κB p65 (P = 0.005, n = 12), indicating activation of the RONS-sensitive NF-κB signalling pathway (Supplementary Figs. 1 and 2).

3.3. Maximal functional reserve

Both sexes showed a similar physiological response to the ISR test when differences in lean mass were accounted for (Table 3). The MFR expressed as total work was similar in males and females when normalized to LLM. This work was developed at a mean intensity above the Wmax in both sexes, with a similar contribution by the glycolytic component of substrate level phosphorylation. Twelve out of 30 males accomplished 6 bouts, i.e., 5 occlusions in the ISR test (8 performed the six bouts at 120% of VO_2max, while 4 did the last bouts at 100% of VO_2max). The rest of the males were able to perform 5 bouts (n = 12) or 4 bouts (n = 6), while 8 females performed 6 bouts (six performed the six bouts at 120% of VO_2max, while two did 4 at 120% and 2 at 100% of VO_2max), 2 females performed 5 bouts, one 4 bouts, and 2 only 3 bouts. The tests with free circulation recovery were carried out by 24 males and 6 females. All subjects could perform the six bouts programmed, except for one female who did five bouts (three at 120% and two at 100% of VO_2max). The total work performed was 67% higher in the test with free circulation during the recovery periods (FCR session) when the same number of bouts performed in the ISR tests were compared (P < 0.001). Subjects were able to perform more bouts and at a higher mean relative intensity when they recovered with free circulation.

Table 3
Ergospirometric variables assessed during the maximal functional reserve test (mean ± SD).

Maximal functional reserve (tests with ischaemic recovery)			
	Males (n = 30)	Females (n = 13)	*p*
Time to exhaustion (s)	77.6 ± 35.1	110.1 ± 69.2	0.129
Power (W)	290 ± 51	203 ± 48	**0.000**
Power (W·kg BW^{-1})	4.0 ± 0.7	3.5 ± 0.4	**0.020**
Power (W·kg LM^{-1})	5.2 ± 0.8	5.1 ± 0.6	0.768
Power (W·kg LLM^{-1})	14.4 ± 2.3	14.5 ± 1.9	0.853
Work (kJ)	22.3 ± 10.4	22.9 ± 15.9	0.899
Work (kJ·kg BW^{-1})	0.30 ± 0.13	0.39 ± 0.26	0.287
Work (kJ·kg LM^{-1})	0.40 ± 0.17	0.56 ± 0.36	**0.046**
Work (kJ·kg LLM^{-1})	1.10 ± 0.48	1.61 ± 1.01	0.598
O_2 demand (mL)	4711 ± 2328	4730 ± 3595	0.984
Accumulated O_2 (mL)	3248 ± 1821	3360 ± 2557	0.471
Accumulated O_2 (mL·kg LLM^{-1})	160 ± 85	236 ± 164	0.674
O_2 deficit (mL)	1463 ± 709	1370 ± 1097	0.746
O_2 deficit (mL·kg BW^{-1})	19.9 ± 8.8	23.0 ± 16.5	0.545
O_2 deficit (mL·kg LM^{-1})	26.1 ± 11.6	33.3 ± 23.3	0.184
O_2 deficit (mL·kg LLM^{-1})	72.4 ± 32.2	95.4 ± 66.4	0.271
O_2/Work (mL·kJ^{-1})	127 ± 28	143 ± 20	0.070
O_2/Power (mL·W^{-1})	14.4 ± 7.0	17.8 ± 11.5	0.348
% Anaerobic Energy	40.2 ± 10.8	33.5 ± 9.8	0.071
RER	1.33 ± 0.11	1.26 ± 0.23	0.183
V_E (L·min^{-1})	114.4 ± 18.6	91.7 ± 17.7	**0.001**
RR (Breaths·min^{-1})	52.2 ± 8.4	57.7 ± 8.9	0.069
V_E/ VO_2	51.7 ± 8.6	52.8 ± 10.3	0.725
V_E/ VCO_2	35.9 ± 4.6	41.4 ± 6.1	**0.003**
$P_{ET}O_2$ (mmHg)	120.9 ± 3.4	121.2 ± 3.9	0.783
$P_{ET}CO_2$ (mmHg)	30.3 ± 3.7	28.2 ± 2.8	0.203
Vastus Lateralis TOI (%)	62.0 ± 3.9	62.3 ± 5.2	0.835
Frontal lobe TOI (%)	63.6 ± 6.1	58.1 ± 8.9	**0.030**
MCAv (cm.s^{-1})	40.6 ± 6.8	40.8 ± 14.6	0.966

HRmax: maximal heart rate; VO_{2max}: maximal oxygen uptake; Wmax: maximal intensity during the incremental exercise test to exhaustion; LLM: lean mass of the lower extremities; Accumulated O_2: total amount of O_2 consumed during the test; and the average values during the 120% CP test for: RER, respiratory exchange ratio; $\dot{V}_E$, pulmonary ventilation; RR, respiratory rate; $P_{ET}O_2$, end tidal O_2 pressure; $P_{ET}CO_2$, end tidal CO_2 pressure; TOI, tissue oxygenation index; MCAv, middle cerebral artery velocity. P values based on two-tailed unpaired t-tests.

There were linear associations between the MFR in kJ·kg LLM^{-1} and the accumulated VO_2 in mL·kg LLM^{-1} (r = 0.97, P < 0.001, n = 42) in the MFR test, the O_2 consumed per watt in mL·W^{-1} (r = 0.90, P < 0.001, n = 42), the O_2 deficit in mL·kg LLM^{-1} in the MFR test (r = 0.87, P < 0.001, n = 42), and the *vastus lateralis* O_2 fractional extraction index (r = 0.37, P = 0.014, n = 36) (Supplementary Figs. 3a–d). The O_2 deficit per kg of LLM in the MFR test was linearly associated with the MAOD (r = 0.34, P = 0.027, n = 43) and the number of bouts performed in the test (r = 0.58, P < 0.001, n = 42) (Supplementary Figs. 3e and f). The *vastus lateralis* O_2 fractional extraction index was inversely associated with the Log of SOD1 protein expression (r = −0.50, P < 0.001, n = 42), Log of Nrf2 total protein expression (r = −0.38, P < 0.012, n = 43), and the Log of Nrf2/Keap1 (r = −0.34, P < 0.026, n = 43).

The power developed per kg of LLM in the functional reserve test was linearly associated with the VO_2max in ml•kg LLM^{-1}•min^{-1} (r = 0.45, P < 0.001, n = 43), the Wmean per kg of LLM in the Wingate test (r = 0.46, P = 0.003, n = 40), the percentage of MHC I (r = 0.31, P = 0.040, n = 43), and the protein expression of COXII (r = 0.31, P = 0.044, n = 43).

3.4. Brain perfusion and oxygenation

No association was observed between brain oxygenation and perfusion, as indicated by the frontal lobe TOI and the MCAv, and the MFR in kJ·kg LLM^{-1}. No significant differences were observed in frontal lobe oxygenation between the ISR and FCR sessions (64.2 ± 5.6 and 65.7 ± 4.8 a.u., n = 25, P = 0.21).

3.5. The O_2 deficit during the maximal functional reserve test is associated with some proteins involved in redox regulation

The O_2 deficit per kg of LLM in the MFR test was linearly associated with the protein expression levels in the *vastus lateralis* of $pSer^{40}$-Nrf2 (r = 0.48, P < 0.001, n = 43) and the Log of SOD1 (r = −0.34, P < 0.030, n = 42). Likewise, capillary blood lactate concentration 5 min after the end of the MFR test was linearly associated with V_E (r = 0.38, P = 0.014, n = 30), and protein expression of catalase (r = 0.38, P = 0.013, n = 30), myoglobin (r = 0.36, P = 0.017, n = 30) and the Log of CQS2 (r = 0.39, P = 0.011, n = 30). Keap1 was associated with the percentage of anaerobic energy yield during the MFR (r = 0.41, P = 0.008, n = 42).

3.6. Main predictors of maximal functional reserve

Multiple regression analysis indicated that the main variables predicting the MFR expressed as total work normalized to LLM were the accumulated O_2 uptake per kg of LLM during the MFR test, which explained 94.1% of the variance; the O_2 deficit per kg of LLM during the MFR test, which explained an additional 2.9% of the variance; the mean power output per kg of LLM during MFR test, which explained an additional 1.3% of the variance, and the percentage of MHC I + IIa which explained 0.3% of the variance (R^2 = 0.985, P < 0.001, Table 4). The accumulated O_2 uptake in the MFR test was predicted by the O_2 deficit per kg of LLM, which explained 59.4% of the variance; SERCA1 protein expression, the frontal lobe oxygenation index, Keap1 protein expression, SERCA2 protein expression, and myoglobin protein expression accounted for 6.7, 6.1, 4.6, 3.2, and 2.8% of the variance,

respectively ($R^2 = 0.83$, $P < 0.001$, Table 4). The O_2 deficit in the MFR test per LLM was predicted by the protein expression levels of $pSer^{40}$-Nrf2, which explained 21.5% of the variance; the MAOD, which explained 14% of the variance, and the Log SOD1 protein expression levels, which explained 7.1% ($R^2 = 0.45$, $P = 0.001$, Table 4). The mean power output in the MFR test expressed as W per kg of LLM was predicted by the aerobic efficiency in the MFR test expressed as mL of O_2 per kJ, which explained 32.7% of the variance, the VO_2max in $mL \cdot kg^{-1} \cdot min^{-1}$, which explained 20.1% of the variance, Keap1 expression levels 5.6%, and SERCA2 protein expression levels, which explained an additional 6.0% of the variance ($R^2 = 0.65$, $P < 0.001$, Table 4).

3.7. Sex differences in muscle phenotype

Females had a higher percentage of MHC I (53.5 ± 11.4 vs. 34.0 ± 12.4%, $P < 0.001$) and higher basal expression of SERCA2 (4.0 ± 1.4 vs 1.5 ± 1.2 a.u., $P < 0.001$) and CSQ2 (1.0 ± 1.3 vs 2.0 ± 0.7 a.u., $P = 0.014$). Although PFKM was 15% higher in males than females, the difference did not reach statistical significance ($P = 0.057$) (Fig. 2, n = 13 and 30, for females and males, respectively, unless otherwise indicated in the figure legend). Representative immunoblots of all proteins studied are depicted in Fig. 3.

4. Discussion

The present investigation shows in humans that the protein expression of enzymes involved in both sensing and counteracting RONS likely play an important role in determining the exercise capacity during high-intensity exercise requiring a marked contribution of glycolysis for muscle energy production. We have demonstrated that the MFR has a finite magnitude ranging from 0.17 to 3.66 $kJ \cdot kg\ LLM^{-1}$ in healthy young humans, with comparable values in males and females. We have also shown that the MFR is associated with both the O_2 consumed and

Table 4
Predictive models for the maximal functional reserve (MFR).

Predictor	Estimate	SE	95% Confidence Interval		t	p	Stand. Estimate	95% Confidence Interval		Model	Model fit measures		
			Lower	Upper				Lower	Upper		R	R2	P
Predictive model for the maximal functional reserve (MFR) expressed as kJ per kg of lower extremities lean mass (LLM)													
Intercept	−0.18099	0.14754	−0.47993	0.11795	−1.23	0.228							
Accumulated VO_2 ($mL \cdot kg\ LLM^{-1}$)	0.00503	0.000206	0.00461	0.00545	24.47	**< .001**	0.8226	0.7545	0.8908	1	0.97	0.941	**< .001**
O_2 deficit ($mL \cdot kg\ LLM^{-1}$)	0.00295	0.000547	0.00185	0.00406	5.4	**< .001**	0.1871	0.1169	0.2572	2	0.985	0.97	**< .001**
Mean power in MFR test ($w \cdot kg\ LLM^{-1}$)	0.04553	0.00703	0.03129	0.05977	6.48	**< .001**	0.1425	0.0979	0.1871	3	0.992	0.983	**< .001**
MHC I + IIa (%)	−0.00451	0.00165	−0.00786	−0.00116	−2.73	**0.01**	−0.056	−0.0977	−0.0144	4	0.993	0.986	**< .001**
Predictive model for the accumulated O_2 uptake during the MFR test expressed as mL per kg of lower extremities lean mass (LLM)													
Intercept	555.8	111.992	327.09	784.52	4.96	**< .001**							
O_2 deficit ($mL \cdot kg\ LLM^{-1}$)	1.77	0.211	1.33	2.2	8.36	**< .001**	0.685	0.5174	0.85211	1	0.771	0.594	**< .001**
Frontal lobe TOI (a.u.)	−5.3	1.566	−8.5	−2.1	−3.38	**0.002**	−0.289	−0.4635	−0.11455	2	0.81	0.655	**< .001**
SERCA1 protein expression (a.u.)	−57.53	25.052	−108.69	−6.37	−2.3	**0.029**	−0.183	−0.345	−0.02022	3	0.85	0.723	**< .001**
Keap1 protein expression (a.u.)	−54.93	25.98	−107.99	−1.87	−2.11	**0.043**	−0.174	−0.3418	−0.00592	4	0.877	0.769	**< .001**
SERCA2 protein expression (a.u.)	13.88	5.284	3.09	24.67	2.63	**0.013**	0.208	0.0464	0.37019	5	0.895	0.801	**< .001**
Myoglobin protein expression (a.u.)	−39.07	17.571	−74.96	−3.19	−2.22	**0.034**	−0.197	−0.3776	−0.01606	6	0.91	0.829	**< .001**
Predictive model for the O_2 deficit during the MFR test expressed as mL per kg of lower extremities lean mass (LLM)													
Intercept	−14.359	20.685	−56.271	27.553	−0.694	0.492							
$pSer^{40}$Nrf2 protein expression (a.u.)	73.477	20.476	31.99	114.965	3.589	**< .001**	0.452	0.197	0.7077	1	0.484	0.234	**0.001**
MAOD ($mL \cdot kg\ LLM^{-1}$)	0.406	0.125	0.152	0.66	3.237	**0.003**	0.399	0.149	0.6494	2	0.612	0.374	**< .001**
Log SOD1 protein expression (a.u.)	−53.736	24.788	−103.961	−3.51	−2.168	**0.037**	−0.274	−0.53	−0.0179	3	0.667	0.445	**< .001**
Predictive model for the mean power in MFR test expressed as W per kg of lower extremities lean mass (LLM)													
Intercept	13.3272	2.66098	7.9355	18.7188	5.01	**< .001**							
O_2 cost, O_2/Work ($mL \cdot kJ^{-1}$)	−0.054	0.00838	−0.071	−0.037	−6.44	**< .001**	−0.652	−0.857	−0.4468	1	0.572	0.327	**< .001**
VO_2max ($mL \cdot kg\ LLM^{-1}$)	0.0611	0.01293	0.0349	0.0873	4.72	**< .001**	0.457	0.2613	0.6536	2	0.727	0.528	**< .001**
Keap1 protein expression (a.u.)	−1.5425	0.56474	−2.6868	−0.3982	−2.73	**0.01**	−0.277	−0.4821	−0.0715	3	0.771	0.594	**< .001**
SERCA2 protein expression (a.u.)	0.3192	0.12625	0.0634	0.575	2.53	**0.016**	0.245	0.0486	0.4407	4	0.809	0.654	**< .001**

MAOD, maximal accumulated oxygen deficit; MCH, myosin heavy chain; Nrf2, nuclear factor (erythroid-derived 2)-like 2; SERCA, sarco-endoplasmic reticulum Ca^{2+} ATPase; Keap1, kelch-like ECH-associated protein 1; SOD, superoxide dismutase; Log, logarithm.

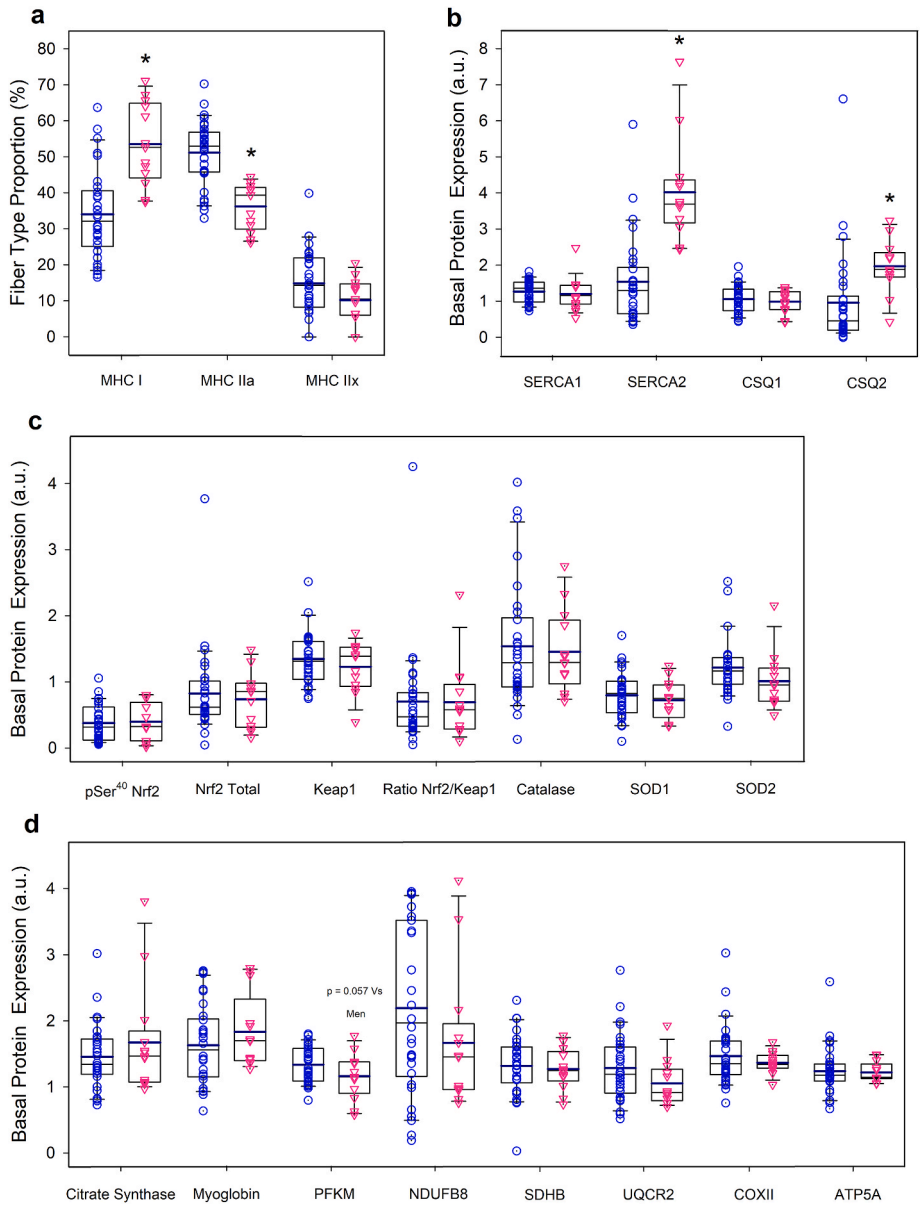

Fig. 2. Sex differences in muscles in myosin heavy chain proportions (a), and basal expression of proteins involved in calcium handling (b), redox regulation (c), and muscle metabolism and O_2 transport (d). n = 43; 30 M and 13 F, except for SOD1 and SOD2 with n = 42. Males: blue circles; females: red triangles. The values shown are means ± standard errors and expressed in arbitrary units (a.u.). * $P < 0.05$ for between-sex differences.

the O_2 deficit observed during the MFR test, indicating that both oxidative and substrate level phosphorylation contribute to this functional reserve. However, conceptually no functional reserve would exist without a metabolic reserve in glycolytic substrate level phosphorylation, since the second exercise bout was always performed at a work rate of 120% of VO_2max. Moreover, even for the bouts carried out at 100% of VO_2max a contribution from substrate level phosphorylation is required to re-start exercising, given the mismatch between O_2 demand and VO_2 at the beginning of exercise. Overall, these results imply that during repeated supramaximal exercise to task failure, a large metabolic reserve exists in both oxidative and substrate level phosphorylation at exhaustion, the latter been explained by its glycolytic component, as previously reported [15,16]. We have also shown that the aerobic component of the MFR is positively associated with the total O_2 deficit and SERCA2 protein expression levels and negatively with the exercising frontal lobe oxygenation and the basal protein expression levels of SERCA1, Keap1, and myoglobin. In turn, the anaerobic component of the MFR is positively associated with the basal protein expression levels of $pSer^{40}$-Nrf2 and the MAOD and negatively with the basal protein expression levels of SOD1. Finally, the mean power output developed during the MFR test was associated positively with the VO_2max in mL•kg LLM^{-1}•min^{-1} and SERCA2 protein expression levels, and negatively with the aerobic cost of exercise in mL of O_2.kJ^{-1} and Keap1 protein expression levels. Globally, these findings indicate that MFR test performance depends on the contribution of anaerobic capacity, which is essential, and the VO_2max which establishes the mean power output that can be developed in the MFR test.

4.1. *Is the functional reserve explained by muscle properties?*

In agreement with our hypotheses, the ISR protocol elicited the phosphorylation of p38 MAPK and NF-κB p65, which depends on exercise-produced RONS [60–63]. Accordingly, the functional reserve and its aerobic and glycolytic components can be predicted by variables intrinsically linked to the muscle phenotype, mostly related to redox regulation and calcium handling. As shown in Table 4, multiple

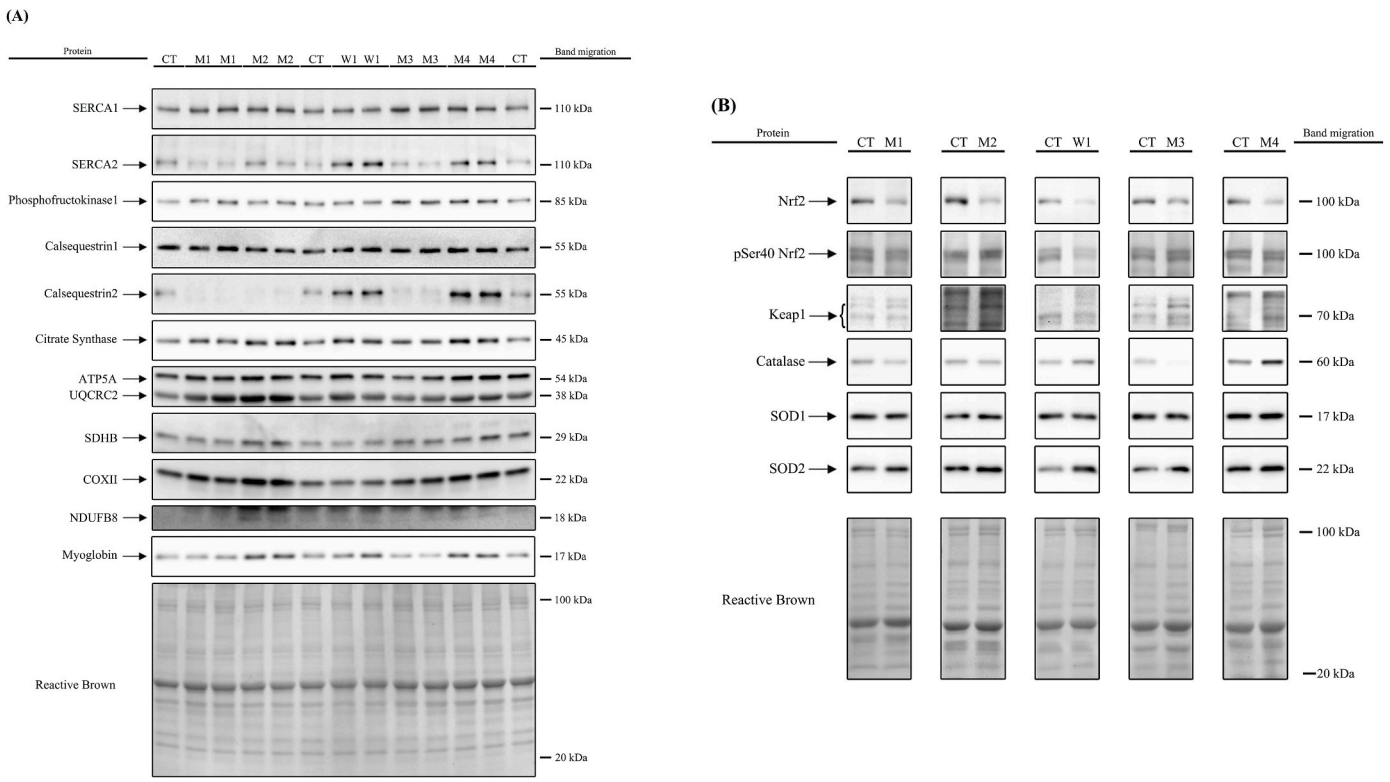

Fig. 3. Representative images of basal protein expression levels (Western Blot) for all proteins studied and total amount of protein loaded (Reactive Brown Staining) from one woman and four men participating in the study. A control human sample (non-experimental) was included onto each gel in triplicate or quadruplicate to allow normalization and as a loading control. Images from top to bottom: SERCA1, SERCA2, phosphofructokinase1, calsequestrin1, calsequestrin2, citrate synthase, ATP5A, UQCRC2, SDHB, COXII, NDUFB8, myoglobin and Reactive Brown (as total protein loading control) with experimental samples run in duplicate (Panel A), and Nrf2, $pSer^{40}$ Nrf2, Keap1, catalase, SOD1, SOD2 and Reactive Brown (Panel B). CON, control non-experimental sample; M, sample indicating a male participant; W, sample indicating a female subject. Estimated molecular weights are indicated on the right side of the blot.

regression analysis indicates that the percentage of MHC I + IIa contributes to predicting the MFR in kJ·kg LLM^{-1}, however, the proportion of variance explained by MHCs is very small compared to the contribution made by the aerobic and the glycolytic components of the MFR, which explain 94 and 3% of the variance in MFR in kJ·kg LLM^{-1}. Nonetheless, the mean power output developed during the MFR test was positively associated with indices of aerobic metabolism with the main role played by the O_2 cost of exercise, which had a negative impact, meaning that the higher the O_2 cost of exercise, the lower the MFR. In contrast, a higher VO_2max predicts a greater power per kg of LLM during the MFR test. These two findings indicate that both the VO_2max and the efficiency with which the O_2 is utilized are crucial determinants of the power that can be developed during the MFR test. Thus, we decided to examine which factors could explain the aerobic and the anaerobic components of the functional reserve.

4.2. A higher functional reserve is mostly explained by a greater capacity to utilize O_2 at exhaustion combined with an enhanced reserve in glycolytic capacity

The present results indicate that the aerobic component of the functional reserve depends on multiple factors defined by intrinsic properties of the exercising muscles like the glycolytic capacity to resynthesize ATP during exercise, the basal expression of the ROS-sensor protein Keap1, the basal protein expression levels of the calcium-regulating proteins SERCA1 and SERCA2, and the basal expression of myoglobin, as commented in the lines that follow. It should be highlighted that the main predictor of the aerobic component of the functional reserve is the O_2 deficit, which represents the contribution to ATP resynthesis made by the glycolytic component of substrate-level phosphorylation. This is explained by the fact that the subjects with a higher O_2 deficit were able to perform more bouts of exercise, i.e., the glycolytic component of the MFR determines the capacity to restart exercising after each occlusion.

Two isoforms of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) protein are present in skeletal muscle and responsible for the active transport of Ca^{2+} from the sarcoplasm to the sarcoplasmic reticulum. SERCA1 is expressed exclusively in fast-twitch fibres, while SERCA2 is the variant encountered in cardiac muscle and slow-twitch fibres [64]. Research has shown increased muscle SERCA1 and SERCA2 content after sprint training [65] and SERCA1 after 23 days of unilateral lower limb suspension [66]. In contrast, reduced SERCA1 [67,68] and SERCA2 [69] have been reported after endurance training in humans. Although an association between reduced SERCAs protein expression and cycling efficiency has been reported [69], no relationship was observed in the current study between VO_2/W during the MFR test and the expression of either SERCA or the combination of the two. Our results show that the aerobic component of the MFR associates positively with the expression of SERCA2 and negatively with the expression of SERCA1, implying that the aerobic component of the MFR is determined by the muscle fibre properties linked to aerobic metabolism and endurance. More specifically, our results suggest that an enhanced capacity to reuptake Ca^{2+} in type I fibres is a contributing factor to the aerobic component of the MFR. Moreover, since high oxidative stress may reduce the SERCAs' activity [70], a higher amount of SERCA2 may allow better resisting oxidative stress's detrimental effects on Ca^{2+} homeostasis in the slow-twitch fibres and explain a higher aerobic MFR as observed in the current experiments.

Another novelty of this study is the measurement of muscle myoglobin content in a large sample of volunteers. The variance in

myoglobin expression explained 2.8% of the variance in the aerobic MFR, being its standardized estimate negative. This seems counterintuitive, given the role of myoglobin in intracellular O_2 diffusion [71]. However, myoglobin overexpression may result in nitrosative stress due to the nitrite reductase activity of myoglobin and a low cellular PO_2, as presumably reached during the occlusions in our experiments [72]. Excessive nitric oxide (NO) may inhibit mitochondrial respiration [73], limiting the aerobic component of the MFR.

Lastly, 2.7% of the variance in the aerobic component of the MFR was explained by Keap1 protein expression, such that the lower the expression of Keap1, the higher the aerobic component of the MFR. Likewise, Keap1 had a negative predictive value on the mean power developed during the MFR test. These findings concur with the enhanced endurance [11] and strength [74] observed in a genetic model of reduced expression of Keap1 in skeletal muscle. We surmise that lower availability of Keap1 in the rested state may facilitate greater levels of $pSer^{40}$-Nrf2 and antioxidant enzymes [74] allowing a more efficient counteraction of RONS production during repeated cycles of ischaemia-reperfusion. This agrees with the observed greater increase in antioxidant proteins in slow than fast-twitch muscles in genetic models of Keap1 deletion [74].

4.3. The glycolytic component of the functional reserve is related to mechanisms regulating the muscle's antioxidant capacity

The O_2 deficit incurred in the MFR can be predicted by the protein expression levels of $pSer^{40}$-Nrf2, which explains 23% of the variance of the MFR. The phosphorylation of Nrf2 at Serine [40] facilitates its translocation to the nucleus and the subsequent gene transactivation [75,76]. Nrf2 can be linked to exercise performance by several mechanisms. First, rodents overexpressing Nrf2 have increased endurance [6,74] and nuclear Nrf2 protein content after prolonged running [7]. Although the molecular mechanisms by which Nrf2 may increase performance remain elusive, they are likely linked to an improved antioxidant capacity [74]. For example, Nrf2 increases the expression of the TP53-Induced Glycolysis and Apoptosis Regulator (TIGAR) [77], which promotes the production of nicotinamide adenine dinucleotide phosphate (NADPH) for glutathione (GSH) regeneration, resulting in enhanced antioxidant capacity in the resting muscle [78]. It has also been shown that Nrf2 elicits the expression of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) [79], which catalyses the conversion of fructose-6-phosphate to fructose-2,6-bisphosphate -a potent allosteric activator of 6-phosphofructokinase-1 (PFK-1). Experiments with cancer cells also indicate that Nrf2 may upregulate the expression of HK2 [80], which may allow higher glycolytic rates. Moreover, increased HK2 has been shown to bind to mitochondria, where it secures a steady ADP formation, contributing to maintaining the membrane potential and reducing ROS production [81].

A higher SOD2 has been shown to protect against ischaemia-reperfusion [82]. In the present exercise model, the skeletal muscle is submitted to repeated episodes of ischaemia with full muscle deoxygenation followed by fast reperfusion upon resumption of exercise. We surmise that SOD2 helps preserve mitochondrial function and therefore enables the utilization of O_2 at close-to-maximal rates even in states of apparently unsurmountable muscle fatigue. This interpretation is also supported by the linear association observed between VO_2max per kg of LLM and SOD2. Moreover, we have recently shown that the oral administration of polyphenols with potent direct (by quenching RONS) and indirect (by inhibiting XO and NOX, two of the main sites of RONS production during high-intensity exercise [3]) antioxidant effects enhance O_2 consumption and performance during repeated sprint exercise when subjects are requested to sprint maximally with normal blood flow after a short period of post-exhaustive exercise ischaemia [17,19].

RONS have been implicated in muscle fatigue [83], and RONS production is facilitated by the acidification caused by strong activation of the glycolysis in the presence of Fe^{2+} through the Fenton reaction [84]. Both catalase and myoglobin may counteract the deleterious effects of RONS produced during high-intensity exercise, particularly when accompanied by high glycolytic rates, as in the present exercise model. This concurs with the association between blood lactate concentration at the end of the MFR and basal catalase and myoglobin protein expressions reported here. Both proteins are crucial in redox regulation in skeletal muscle [85–88].

Since part of the functional reserve could be explained by mechanisms delaying central fatigue including the agonistic capacity to overcome fatigue, we also examined whether differences in brain oxygenation could explain our findings.

4.4. Brain and vastus lateralis oxygenation role in the MFR

Brain oxygenation depends on arterial O_2 content, cerebral blood flow and cerebral metabolic rate. In the present investigation, frontal lobe oxygenation explained 6.1% of the variability in the aerobic component of MFR. We hypothesized that subjects with higher brain oxygenation levels would perform better during repeated supramaximal exercise to exhaustion. Paradoxically, the results point in the opposite direction, as indicated by the negative coefficient (see the corresponding standardized estimate in Table 4). The latter implies that subjects with a higher MFR achieve greater levels of brain deoxygenation during intense exercise. Thus, we believe that the lower frontal lobe oxygenation observed in the subjects with a greater aerobic component of the functional reserve is a consequence and not the cause since humans can tolerate much lower levels of brain oxygenation during sprint exercise in severe acute hypoxia when assessed using the same equipment and laboratory conditions than in the present experiments [50].

However, the TOI *vastus lateralis* O_2 extraction index was linearly associated with the MFR in kJ·kg LLM^{-1}. The TOI *vastus lateralis* O_2 extraction index depends on the balance between O_2 delivery and O_2 utilization, such that a higher muscle perfusion for a given muscle O_2 demand with results in a greater the TOI *vastus lateralis* O_2 extraction index during the MFR test, facilitating both O_2 delivery and removal of metabolites to attenuate fatigue.

4.5. The functional reserve is similar in males and females matched for aerobic and anaerobic fitness

In the present investigation males and females had a similar VO_2max normalized to LLM, indicating similar aerobic fitness. Likewise, no sex differences were observed in anaerobic power and capacity, as reflected by similar peak and mean power output per kg of LLM in the Wingate test, as well as MAOD values normalized to LLM and blood lactate concentrations after the MFR test. In our previous study we observed that males and females achieved similar values of functional reserve in kJ·kg LLM^{-1} after two bouts of repeated supramaximal exercise with total occlusion of the circulation during the recovery periods [16]. The present investigation confirms our previous findings and shows that when exercise bouts are repeated until the subjects cannot perform an additional bout, males and females have a similar functional reserve. This occurred even though females had a higher percentage of MHC I and higher basal expression of SERCA2 and CSQ2 in their *vastus lateralis* than males, while no significant sex-differences were observed in the rest of muscle proteins analysed in the present investigation.

4.6. Limitations

In the present investigation substrate-level phosphorylation has been assessed indirectly, assuming constant muscle efficiency during high-intensity exercise [54,89]. Although a decreased muscle efficiency with fatigue has been reported *in vitro* [90], the impact of fatigue on muscle efficiency in human skeletal muscle is not conclusive [91]. Moreover, at physiological muscle temperatures the effect of fatigue on

muscle efficiency is likely small in humans [15,89,92]. Had muscle efficiency deteriorated with fatigue, we could have underestimated the actual contribution of substrate level phosphorylation to the functional reserve.

Twenty of our volunteers were able to perform six bouts of exercise, i.e., they showed a functional reserve after five occlusions. Thus, the actual functional reserve in these subjects could have been underestimated. Since the contribution of the last bout to the overall functional reserve was negligible in most subjects, and several did the last bout at 100% of VO_2max, we surmise that this potential underestimation would be negligible. Nevertheless, it would be worthwhile to extend the number of repetitions in future experiments until all subjects cannot restart pedalling at 100% of VO_2max.

Finally, this study is the first to analyse the skeletal muscle responses to repeated episodes of ischaemia-reperfusion in humans. Therefore, only young healthy subjects were included, which were quite homogeneous regarding physical fitness. It remains unknown whether elite athletes have increased (or decreased) functional reserve and how ageing and diseases could affect the maximal functional reserve.

In summary, a large metabolic reserve exists in skeletal muscle at exhaustion which has an obligatory component relying on a reserve in glycolytic capacity and a putative component which depends on oxidative phosphorylation. We have shown that this functional reserve is similar in males and females matched for physical fitness. Moreover, we have identified several phenotypic muscle characteristics that explain the aerobic and glycolytic components of the functional reserve and found that the MFR is associated with muscle characteristics determining antioxidant signalling, calcium handling, and O_2 transport and diffusion. Further manipulation of these phenotypic features, through, for example, exercise training/detraining or administering antioxidants, will be required to gain insight into the mechanisms determining the maximal functional reserve.

Disclosure summary

The authors have nothing to disclose.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that support the findings of this study are not openly available due to reasons of sensitivity and are available from the corresponding author upon reasonable request

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2023.102859.

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ESTUDIO 2

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Antioxidant enzymes and Nrf2/Keap1 in human skeletal muscle: Influence of age, sex, adiposity and aerobic fitness

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ABSTRACT

Ageing, a sedentary lifestyle, and obesity are associated with increased oxidative stress, while regular exercise is associated with an increased antioxidant capacity in trained skeletal muscles. Whether a higher aerobic fitness is associated with increased expression of antioxidant enzymes and their regulatory factors in skeletal muscle remains unknown. Although oestrogens could promote a higher antioxidant capacity in females, it remains unknown whether a sex dimorphism exists in humans regarding the antioxidant capacity of skeletal muscle. Thus, the aim was to determine the protein expression levels of the antioxidant enzymes SOD1, SOD2, catalase and glutathione reductase (GR) and their regulatory factors Nrf2 and Keap1 in 189 volunteers (120 males and 69 females) to establish whether sex differences exist and how age, VO_2max and adiposity influence these. For this purpose, *vastus lateralis* muscle biopsies were obtained in all participants under resting and unstressed conditions. No significant sex differences in Nrf2, Keap1, SOD1, SOD2, catalase and GR protein expression levels were observed after accounting for VO_2max, age and adiposity differences. Multiple regression analysis indicates that the VO_2max in mL.kg LLM^{-1}.min^{-1} can be predicted from the levels of SOD2, Total Nrf2 and Keap1 ($R = 0.58$, $P < 0.001$), with SOD2 being the main predictor explaining 28 % of variance in VO_2max, while Nrf2 and Keap1 explained each around 3 % of the variance. SOD1 protein expression increased with ageing in the whole group after accounting for differences in VO_2max and body fat percentage. Overweight and obesity were associated with increased $pSer^{40}$-Nrf2, $pSer^{40}$-Nrf2/Total Nrf2 ratio and SOD1 protein expression levels after accounting for differences in age and VO_2max. Overall, at the population level, higher aerobic fitness is associated with increased basal expression of muscle antioxidant enzymes, which may explain some of the benefits of regular exercise.

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1. Introduction

Ageing, a sedentary lifestyle and obesity are associated with increased oxidative stress, a condition characterized by the oxidation of cellular structures due to an imbalance between oxidant and antioxidant mechanisms [1]. Regular exercise, via hormetic processes elicited by reactive oxygen and nitrogen species (RONS) bursts generated during muscle contractions, may cause an elevation of some antioxidant enzymes [2–4]. However, whether a greater enzymatic antioxidant capacity is necessary to reach a higher VO_2max remains unknown.

Abbreviations

AREs	antioxidant response elements
BMI	body mass index
CAT	catalase
GPx	glutathione peroxidase
GR	glutathione reductase
HRmax	maximal heart rate
Keap1	kelch-like ECH-associated protein 1
LLM	lower extremities lean mass
MHC I	myosin heavy chain type I
MHC IIa	myosin heavy chain type IIa
MHC IIx	myosin heavy chain type IIx
Nrf2	nuclear factor (erythroid-derived 2)-like 2
PKC	protein kinase C
RONS	reactive oxygen and nitrogen species
ROS	reactive oxygen species
SODs	superoxide dismutase
VO_2	oxygen uptake
VO_2max	maximal oxygen uptake
W	watts
Wmax	peak power output during the incremental exercise test to exhaustion

The antioxidant system comprises both enzymatic and nonenzymatic antioxidants. The most abundant enzymatic antioxidants in skeletal muscle are superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase [5]. Muscle fibres express the cytosolic (SOD1, 65–85 % of the total SOD activity present in skeletal muscle) and the mitochondrial (SOD2, 15–35 % of the total SOD activity present in skeletal muscle) isoforms of SOD [5]. The SODs catalyse the dismutation of O_2^- to produce H_2O_2, an oxidant converted into H_2O by the action of GPx and catalase [5], while glutathione reductase (GR) plays a critical role in free radical scavenging by catalysing the reduction of oxidized glutathione (GSSG) back to its active and reduced form (GSH).

These antioxidant enzymes are regulated by the nuclear factor erythroid-derived 2-like 2 (Nrf2) [6–8]. Nrf2 is a transcription factor that, under unstressed conditions, binds to Kelch-like ECH-associated protein 1 (Keap1), an adaptor protein for a ubiquitin E3 ligase complex that ubiquitinates Nrf2 for proteasomal degradation [9]. Thus, in unstressed situations, most Nrf2 is tagged for degradation. Under oxidative or electrophilic stress, Keap1 undergoes a conformational change that stabilizes the Keap1-Nrf2 complex, preventing the degradation of Nrf2 [10]. When the amount of free Keap1 is low, the newly synthetized Nrf2 remains free, and it moves into the nucleus, where it interacts with specific DNA sequences called antioxidant response elements (AREs), triggering the transcription of antioxidant genes [9,10]. Nrf2 can be phosphorylated at Ser^{40} by several ROS-sensitive kinases facilitating Nrf2 release from the Nrf2-Keap1 complex and its subsequent translocation to the nucleus for interaction with AREs [11].

By upregulating the antioxidant capacity of muscles, regular exercise may prevent oxidative stress [12]. Oxidative stress may be caused by increased production of RONS, reduced availability of antioxidants, or a combination of the two [5]. Oxidative stress is facilitated by sedentarism and has been reported in association with ageing, obesity, endothelial dysfunction, hypertension, insulin resistance and cancer [13–17]. However, subjects with better mitochondrial function and higher aerobic fitness are less prone to suffer the detrimental effects of oxidative stress [5]. Thus, part of the benefits of exercise in reducing the incidence of chronic diseases and facilitating healthy ageing may be related to its effects on the antioxidant enzymes and their regulatory factors. Aerobic fitness can be assessed by measuring maximal oxygen uptake (VO_2max), which provides an integrated assessment of the cardiorespiratory system and the capacity of the muscle to extract and utilize O_2. A high VO_2max is a distinctive characteristic of elite endurance athletes, whilst a low VO_2max is associated with shortened life expectancy [18] and increased mortality in clinical populations [19]. A low VO_2max may be due to a reduced O_2 delivery, as observed in patients with cardiorespiratory diseases or diminished O_2 utilization capacity due to a reduced muscle mass, capillarization or mitochondrial respiratory capacity, or a combination of them [20,21]. Despite the lower blood haemoglobin concentration in females, VO_2max values are similar in males and females when normalized to the lean mass of the lower extremities [22]. This is likely explained by females' higher O_2 extraction capacity, attributed to their superior mitochondrial respiratory capacity [23]. Some mitochondrial respiratory complexes and dehydrogenases generate O_2^-, which may damage mitochondrial structures and enzymes and hamper mitochondrial respiration and ATP generation [24]. The latter is efficiently prevented by mitochondrial and sarcoplasmic superoxide dismutases [5]. Research to date has not yet determined if a high level of mitochondrial antioxidant enzymes may be required for a high VO_2max.

Table 1
Physical characteristics, performance, and myosin heavy chain myosin composition (mean ± SD).

	Males (n = 120)	range	Females (n = 69)	range	*P*
Age (years)	31.4 ± 10.5	65.2–18.6	33.0 ± 10.5	54.9–18.2	0.311
Height (cm)	177.6 ± 7.4	198.0–161.0	163.6 ± 6.4	180.0–150.0	**0.000**
Weight (kg)	90.9 ± 18.0	136.9–55.9	81.5 ± 17.3	126.1–41.3	**0.001**
Body fat (%)	28.9 ± 9.4	45.6–7.7	42.5 ± 8.1	54.6–21.5	**0.000**
BMI ($kg.m^{-2}$)	28.7 ± 4.9	41.3–18.8	30.3 ± 5.7	44.7–16.2	**0.045**
Legs' lean mass (kg)	21.8 ± 2.9	30.0–15.1	15.7 ± 2.5	20.8–9.6	**0.000**
HRmax (beats. min^{-1})	187.4 ± 12.3	206.6–134.0	185.8 ± 13.1	212.0–149.0	0.443
VO_2max (mL. kg^{-1}. min^{-1})	38.0 ± 9.7	60.8–20.6	27.5 ± 7.5	47.6–12.6	**0.000**
VO_2max (mL. kg LLM^{-1}. min^{-1})	154.5 ± 26.9	224.8–106.3	137.9 ± 24.1	201.4–82.8	**0.000**
Wmax (W)	256.0 ± 42.4	380.0–157.3	170.5 ± 33.9	257.8–87.0	**0.000**
MHC I (%)	42.1 ± 15.1	76.6–16.5	53.5 ± 11.4	71.2–37.5	**0.013**
MHC IIa (%)	44.8 ± 11.8	70.3–15.7	36.3 ± 6.5	44.5–26.2	**0.015**
MHC IIx (%)	13.1 ± 10.3	39.9–0.0	10.2 ± 6.3	20.6–0.0	0.339

HRmax: maximal heart rate; VO_2max, maximal oxygen uptake; Wmax: maximal intensity during the incremental exercise test to exhaustion; LLM: lean mass of the lower extremities; MHC, myosin heavy chain composition (n = 57 for males, and 13 for females). P values based on two-tailed unpaired t-tests.

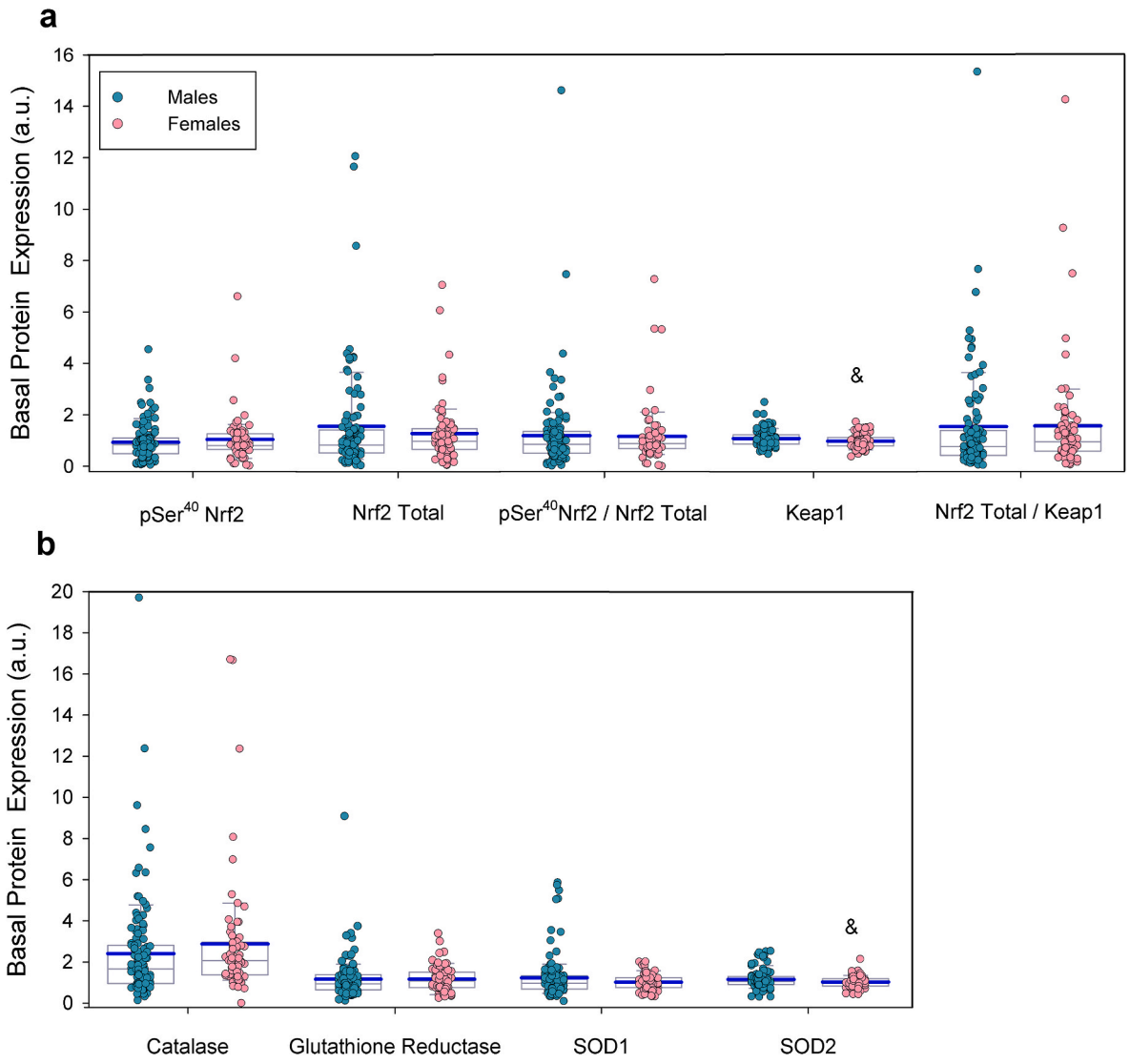

Fig. 1. Sex-related differences in protein expression levels of: pSer^{40}-Nrf2, total Nrf2, pSer^{40}-Nrf2/Nrf2 ratio, Keap1, and Nrf2/Keap1 ratio (a); catalase, glutathione reductase, superoxide dismutase 1 (SOD1), and superoxide dismutase 2 (SOD2) (b). n = 189 (120 males and 69 females) expressed in arbitrary units (a.u.). Box and whisker plots: the extremes of the whiskers represent the limits of the 5th and 95th percentiles, respectively; the thick and thin horizontal lines inside the boxes correspond to the mean and median values, respectively; and the lower and upper limits of the box delimit the 1st and 3rd quartiles, respectively. Male data are presented in green while female data are presented in pink. & $P < 0.05$ males compared to females. Statistical differences were not significant after accounting for differences in age and VO_2max in mL.kg lower extremities lean mass^{-1}.min^{-1}.

A previous study has reported a positive correlation between VO_2max in mL.kg^{-1}.min^{-1} and the skeletal muscle enzymatic activities of SOD and catalase in 12 males aged between 17 and 19 years [25]. These results need confirmation in a larger population sample, including males and females. Moreover, no study has determined if the basal levels of Nrf2 and Keap1 protein expression in human skeletal muscle are linked to VO_2max or are more influenced by other factors such as age, adiposity, or sex. It remains unknown whether there is sexual dimorphism in the expression of antioxidant enzymes in human skeletal muscle, as reported for some tissues in rodents [26]. In support, oestradiol stimulates the expression of enzymatic antioxidants in some cells [27,28], through an Nrf2-depending mechanism [29,30].

Thus, the primary aim of this study was to determine whether higher levels of VO_2max require an increased antioxidant capacity in human skeletal muscle, as assessed by examining the protein expression levels of Nrf2/Keap1 and antioxidant enzymes like SOD, catalase and GR in human skeletal muscle. A secondary aim was to determine whether there is a sexual dimorphism in the expression levels of Nrf2/Keap1 and antioxidant enzymes in human skeletal muscle. For this purpose, we obtained muscle biopsies and measured VO_2max and body composition in 189 volunteers to calculate the VO_2max per kg of lower extremities lean mass (LLM), a variable independent of body size and adiposity. Our central hypothesis is that a greater VO_2max and female sex would be associated with a higher expression of Nrf2 and antioxidant enzymes.

2. Materials and methods

2.1. Subjects

This is a study combining muscle biopsies obtained in previous research projects collected from 189 volunteers, 120 males and 69 females [31–36]. All volunteers were non-smokers. Among them, 55 were healthy university students with varying physical activity levels, while the remaining 148 predominantly led sedentary lifestyles and were either overweight or obese. Within the overweight or obese participants, hypertension (defined as a systolic blood pressure >130 or a diastolic >80 mmHg) was present in 27 of them. Four hypertensives were on diuretics, three on RAS inhibitors/blockers, and one was treated with a

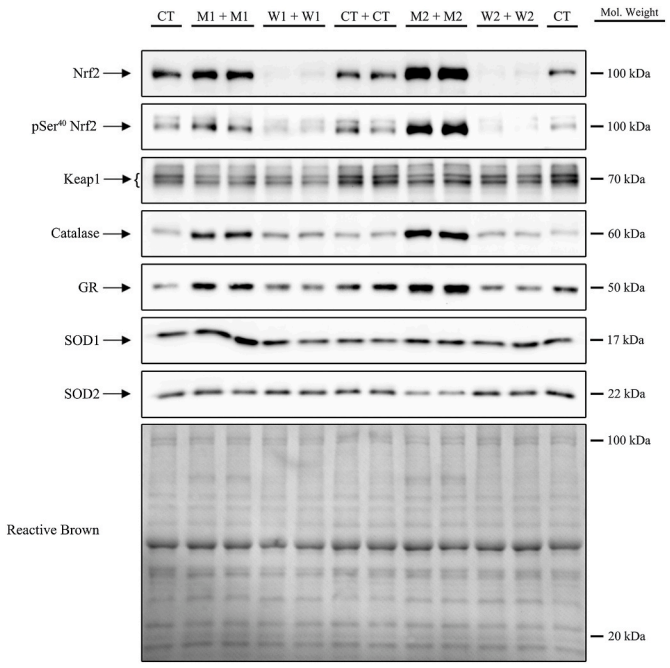

Fig. 2. Representative visual depictions of baseline protein expression levels were obtained using Western Blot analysis for the proteins under investigation. The total protein loaded onto the gels was evaluated through Reactive Brown Staining (last image). The figure represents 2 male and 2 female individuals. To ensure normalization and serve as a control for loading consistency, a non-experimental human sample was included in quadruplicate on each gel. The images presented in a top-to-bottom sequence encompass total Nrf2, $pSer^{40}$-Nrf2, Keap1, catalase, SOD1, SOD2, and Reactive Brown. The reference 'CT' denotes the control non-experimental sample, while 'M' and 'W' respectively indicate samples obtained from male and female participants. Molecular weights are displayed on the right side of the blot.

calcium antagonist. One participant had type 2 diabetes handled with diet and exercise, while two were on statins, as previously reported [37].

All female participants were premenopausal, as pre-established by the inclusion criteria of the original studies. Of these, three were using oral contraceptives. The experiments were conducted per the Declaration of Helsinki, except for registration in a database. All subjects signed a written informed consent before the start of the procedures.

2.2. Main procedures

All volunteers were instructed to abstain from exercise, alcohol, and caffeine intake 48 h before testing and muscle biopsy procedures. Additionally, participants were requested to refrain from taking nutritional supplements or vitamins throughout the study. Before initiating the experiments, the subjects were familiarized with the exercise tests. Subsequently, their anthropometric characteristics were documented, and body composition was assessed using dual-energy X-ray absorptiometry (Lunar iDXA, General Electric, Madison, WI, USA) [38]. Then, their VO_2max and maximal power output (Wmax) were determined using an incremental cycle ergometer exercise test (Lode Corival/Excalibur Sport, Groningen, The Netherlands). The incremental exercise test was customized to the volunteers' profile, with load increments designed to bring exhaustion between 6 and 20 min [39]. Oxygen uptake (VO_2) during all the exercise tests was monitored through open-circuit indirect calorimetry using metabolic carts (Vyntus, Jaeger-CareFusion, Hoechberg, Germany; Vmax N29, Sensormedics, Yorba Linda, CA, USA; COSMED, Rome, Italy; and Jaeger Oxycon Pro, Viasys Healthcare, Hoechberg, Germany) in breath-by-breath mode. Prior to each test, the gas analysers were calibrated following the manufacturer's guidelines. Respiratory variables were recorded breath-by-breath and averaged every 20 s. The peak 20-s averaged VO_2 was retained as the VO_2max and expressed per kg of leg lean mass.

2.3. Muscle biopsies

All participants were asked to refrain from intense physical exertion for a period of 48 h prior to the muscle biopsy, which was conducted following an overnight fast of 10–12 h. They were also instructed to avoid consuming carbonated, caffeinated and alcohol-containing beverages for 24 h before the tests. Muscle biopsies were obtained from the middle section of the muscle *vastus lateralis* muscle using Bergstrom's technique with suction. For this purpose, the skin was disinfected, and the skin and subcutaneous adipose tissue was infiltrated with local anaesthetic (1–2 mL of Lidocaine 2 % without epinephrine). Special care was taken to avoid injecting lidocaine beneath the superficial fascia. After a pause of 10 min, an incision of 6–7 mm was made, and the biopsy needle was inserted 2 cm deep into the muscle belly and 4 cm apart from the point of local anaesthesia. The procured muscle sample, approximately 100 mg, was meticulously rid of debris and fat tissue and subsequently flash-frozen in liquid nitrogen and preserved at −80 °C until further analysis.

Muscle lysates were prepared as described previously [40], and total protein concentration was quantified in triplicate using the bicinchoninic acid assay [41]. For this purpose, 10 mg of muscle were homogenized in urea lysis buffer (6 M urea, 1 % SDS), 50X Complete protease inhibitor and 10X PhosStop phosphatase inhibitor cocktails (Roche). Afterwards, the lysate was centrifuged for 12 min at 25,200 g at 16 °C and the obtained supernatant containing the protein fraction was diluted with electrophoresis loading buffer (160 mM Tris-HCl, pH 6.8, 5.9 % SDS, 25.5 % glycerol, 15 % β-mercaptoethanol- bromophenol blue). The amount of protein required for optimal resolution of the western blot analysis was determined by loading a gradient of control protein extracts (non-interventional human muscle prepared similarly to the experimental samples) in different amounts ranging from 1 to 35 μg, assayed with different antibody concentrations. Equal amounts of protein (1.5–15 μg), corresponding to the middle section linear antigen-antibody response, were loaded and electrophoresed with SDS-PAGE using the system of Laemmli [42]. Then, the proteins were transferred onto the polyvinylidene fluoride (PVDF) membranes for protein blotting (Bio-Rad Laboratories, Hercules, CA, USA). The samples from each subject were run onto the same gel with an equal protein amount from an internal control (same used for linearity optimization) loaded in triplicate or quadruplicate. The densitometric value of the protein of interest was normalized to the mean value of the control sample to account for between-gels variability.

The membranes were subjected to blocking for 1 h using either 4 % bovine serum albumin (BSA) or 2.5–5% non-fat dried milk powder (blotting-grade blocker), diluted in Tris-buffered saline that included 0.1 % Tween 20 (TBS-T). The membranes were incubated overnight at 4 °C with primary antibodies diluted using either the 4 % BSA-blocking buffer or the 5 % Blotto-blocking buffer. Following the incubation with primary antibodies, the membranes were washed and then incubated at room temperature with either an HRP-conjugated anti-rabbit or anti-mouse antibody. In all cases, these were diluted at ratios ranging from 1:5000 to 1:20000 with a 5 % Blotto-blocking buffer. The membranes were then exposed to chemiluminescent visualization with Clarity™ Western ECL Substrate (Bio-Rad Laboratories) using the ChemiDocTM Touch Imaging System (Bio-Rad Laboratories). The optical density of the bands was measured using the Image Lab© software 5.2.1 (Bio-Rad Laboratories). Equal loading and transfer efficiency was verified by staining with Reactive Brown 10 (Sigma-Aldrich, St. Louis, MO, USA) [37].

The Protein Plus Precision All Blue Standards were procured from Bio-Rad Laboratories (Hemel Hempstead Hertfordshire, UK). The antibodies employed in this investigation were obtained from different manufacturers. The corresponding catalogue numbers from Abcam

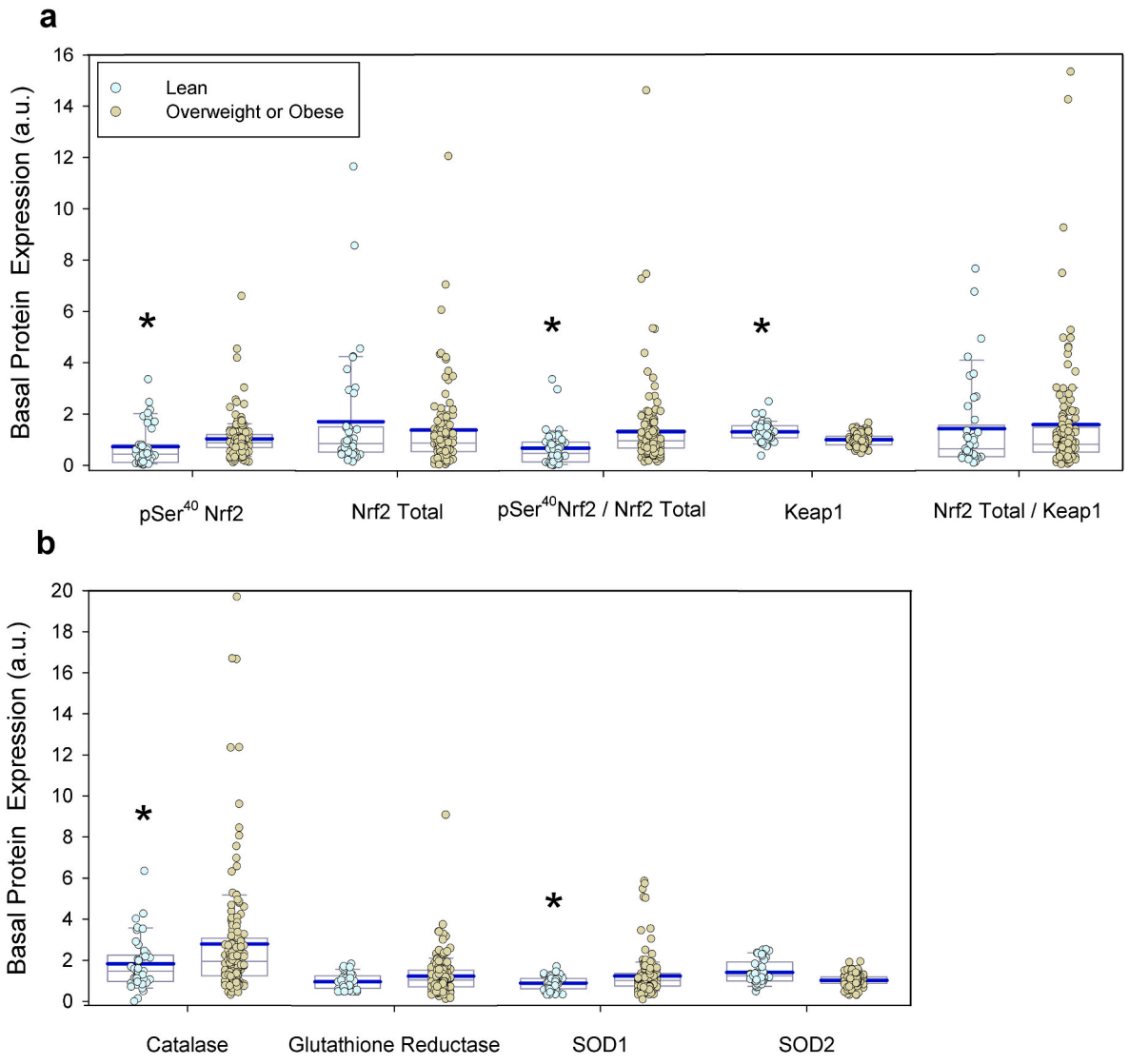

Fig. 3. Protein expression levels in lean and overweight or obese participants. Nrf2 total, pSer^{40}-Nrf2, pSer^{40}-Nrf2/Nrf2 ratio, Keap1, and Nrf2/Keap1 ratio (a); catalase, glutathione reductase, superoxide dismutase 1 (SOD1), and superoxide dismutase 2 (SOD2) (b). n = 189 (41 lean and 148 with overweight or obesity). Box and whisker plots: the extremes of the whiskers represent the limits of the 5th and 95th percentiles, respectively; the thick and thin horizontal lines inside the boxes correspond to the mean and median values, respectively; and the lower and upper limits of the box delimit the 1st and 3rd quartiles, respectively. Data from lean participants are presented in blue while data for overweight or obese participants are presented in yellow. *P < 0.05 lean compared with overweight or obese participants.

(Waltham, MA, USA) were as follows: pSer^{40}-Nrf2 (no. ab76026), Nrf2 (no. ab62352), Keap1 (no. ab119403) and SOD1 (no. ab16831). The antibodies purchased from Cell Signaling Technology (Denver, MA, USA) were catalase (no.14097) and SOD2 (no. 13141). The antibody for GR was purchased from Proteintech (Rosemont, IL, USA) (no. 18257-1-AP). The secondary HRP-conjugated goat anti-rabbit (no. 111-035- 144) and the HRP-conjugated goat anti-mouse (no.115-035-003) antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA). Additional secondary HRP-conjugated goat antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA): anti-rabbit (no. sc2004) and anti-mouse (no. sc2031). See Supplementary Table 1 for a more detailed description of the antibodies and procedures.

2.4. Myosin heavy chain analysis

Myosin heavy chain isoform proportions were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the same aliquots used for western blotting. Experimental and two control samples (7.5–10 μg) were loaded in triplicate onto the same gel. The two control samples served as an internal control to assess the inter-gel variability but were not used for normalization purposes. The experimental samples and controls were processed at 4 °C on an SDS-PAGE gel with a 3 % acrylamide (v/v) phase (stacking gel) for close to 12 h at 70 V, and subsequently, on a 6 % acrylamide (v/v) and 30 % glycerol (v/v) phase (resolving gel) for about 20 h at 350 V. The gels were then stained with Coomassie for roughly an hour, followed by an hour of distaining using a solution of 40 % methanol (v/v) and 10 % glacial acetic acid (v/v). The gels were then submerged in distilled water for around an hour for background subtraction. Finally, the MHC isoform content was determined by scanning the gel (GS-800 Imaging Densitometer, Bio-Rad Laboratories, Hercules, CA, USA) and followed by quantification with the Image Lab© software 5.2.1 (Bio-Rad Laboratories).

2.5. Statistics

The Shapiro-Wilks test was employed to verify the Gaussian distribution of variables. Sex differences for ergometric variables and physical

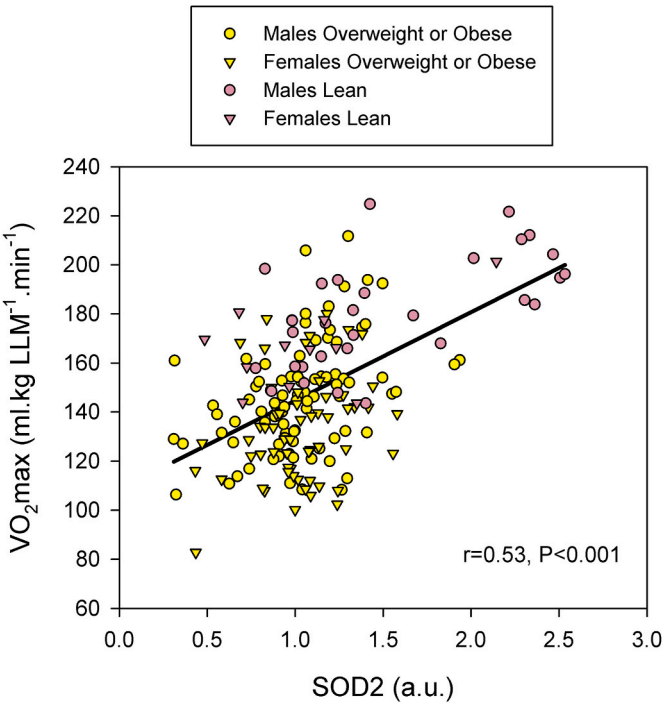

Fig. 4. Relationship between VO_2max in mL.kg of lower extremities lean mass^{-1}.min^{-1} and protein expression of superoxide dismutase 2 (SOD2). n = 188 (41 lean and 147 with overweight or obesity). Data from lean participants are presented in magenta while data for overweight or obese participants are presented in yellow, including a circle shape for males and a triangle shape for females. The values shown are means ± standard errors and expressed in mL.kg LLM^{-1}.min^{-1} and arbitrary units (a.u.). Statistical significance was set at P < 0.05.

characteristics were determined using t-tests for independent samples. Sex differences in protein expression levels were determined by ANOVA, testing the impact of VO_2max in mL.kg LLM$^{-1.min^{-1,}}$ age, and percentage of body fat. Simple and multiple linear regression analyses were used to explore linear relationships between variables. Unless otherwise mentioned, results are presented as the mean ± the standard deviation (SD). A P < 0.05 was considered statistically significant. Statistical analyses were conducted using IBM SPSS Statistics v.29 for Mac (SPSS Inc., Chicago, IL, USA) and Jamovi v1.8.1. (Jamovi project, 2021).

3. Results

3.1. Physical characteristics and performance

Males and females had similar ages, but females had a lower body weight, leg lean mass, and a higher percentage of body fat (Table 1). Males had a 38 and 12 % higher VO_2max per kg of body weight and kg of LLM, respectively (both P < 0.001) (Table 1). Myosin heavy chain composition also differed between sexes, with females exhibiting relatively more MHC I and less MCH IIa (Table 1).

3.2. Sex differences in muscle antioxidant proteins and Nrf2/Keap1

Fig. 1 shows no significant between-sex differences in pSer^{40}-Nrf2, Total Nrf2, pSer^{40}-Nrf2/Total Nrf2 ratio, and Nrf2/Keap1 ratio, catalase, GR and SOD1. Although the Keap1 protein expression was 22 % higher in males than females (P = 0.039) (Fig. 1a), the Nrf2/Keap1 ratio was similar in both sexes. Males also had 13 % higher SOD2 protein expression levels than females (P = 0.032) (see representative immunoblots in Fig. 2). However, the sex difference in Keap1 and SOD2 disappeared after accounting for differences in VO_2max expressed as mL.kg LLM^{-1}.min^{-1}, or after accounting for VO_2max and age, or VO_2max, age and body fat percentage as covariates.

3.3. Age

Age was linearly associated with VO_2max in mL.kg LLM^{-1}.min^{-1} (r = −0.38, P < 0.001, n = 189), Keap1 (r = −0.35, P < 0.001, n = 189), SOD1 (r = 0.25, P < 0.001, n = 189), and SOD2 (r = −0.16, P < 0.001, n = 189). After accounting for the percentage of body fat and VO_2max, the association between age and Keap1 (r = −0.21, P = 0.005, n = 189) and age and SOD1 (r = 0.29, P < 0.001, n = 189) remained statistically significant.

3.4. Overweight and obesity

Among all participants, 148 (90 males and 58 females, were overweight or obese, i.e., BMI ≥ 25 kg m^{-2}), while obesity (BMI ≥ 30 kg m^{-2}) was present in 80 volunteers (43 males and 37 females). No significant differences were observed between participants with or without obesity in Nrf2, Keap1 and the rest of the antioxidant proteins assessed. Catalase protein expression was 9 % higher in the subjects with obesity, after accounting for differences in VO_2max and age (P = 0.046). As illustrated in Fig. 3, when the analysis was factored according to the presence of overweight or obesity, the participants with overweight or obesity had 22 % lower Keap1 and 81 % higher pSer^{40}-Nrf2 protein expression values than their lean counterparts after accounting for differences in age and VO_2max (P < 0.001 and P = 0.012, respectively). SOD1 protein expression was 56 % higher (P = 0.026), and the ratio pSer^{40}-Nrf2/Total Nrf2 was 2.6-fold higher in the group with overweight or obesity (P = 0.014), after accounting for differences in VO_2max and age. In females, the percentage of body fat was associated with the protein expression levels of pSer^{40}-Nrf2 (r = 0.42, P < 0.001, n = 69), Keap1 (r = −0.37, P = 0.002, n = 69), ratio Nfr2/Keap1 (r = 0.28, P = 0.021, n = 69), SOD1 (r = 0.32, P = 0.008, n = 69), catalase (r = 0.35, P = 0.004, n = 69), and GR (r = 0.28, P = 0.021, n = 69). In males, the percentage of body fat was associated with the protein expression levels of Keap1 (r = −0.40, P < 0.001, n = 119) and SOD2 (r = −0.60, P < 0.001, n = 119).

Table 2
Predictive models for VO_2max in mL.kg LLM^{-1}.min^{-1}.

Predictive model for the VO_2max expressed as mL per kg of lower extremities lean mass (LLM)													
			95 % Confidence Interval					95 % Confidence Interval			Model fit measures		
Predictor	Estimate	SE	Lower	Upper	t	p	Stand. Estimate	Lower	Upper	Model	R	R^2	*P*
Intercept	100.83	6.972	87.072	114.59	14.46	<.001							
SOD2 (a.u.)	29.49	4.189	21.229	37.76	7.04	<.001	0.436	0.3136	0.558	1	0.53	0.28	**< .001**
Keap1 (a.u.)	15.75	5.359	5.176	26.32	2.94	0.004	0.179	0.059	0.3	2	0.56	0.32	**< .001**
Total Nrf2 (a.u.)	1.62	0.703	0.237	3.01	2.31	0.022	0.138	0.0202	0.256	3	0.58	0.34	**< .001**
Sex (1 = male; 2 = female)	−10.86	3.354	−17.479	−4.25	−3.24	0.001	−0.4	−0.6437	−0.156	4	0.61	0.37	**< .001**

SOD2, superoxide dismutase 2; Keap1, kelch-like ECH-associated protein 1; Nrf2, nuclear factor (erythroid-derived 2)-like 2.

3.5. VO_2max and antioxidant enzymes

VO_2max expressed as mL.kg $LLM^{-1}.min^{-1}$ was linearly associated with SOD2 ($r = 0.53$, $P < 0.001$) (Fig. 4), Keap1 ($r = 0.30$, $P < 0.001$), and total Nrf2 ($r = 0.21$, $P = 0.003$). However, no significant association was observed between VO_2max expressed as mL.kg $LLM^{-1}.min^{-1}$ and SOD1 ($r = 0.14$, $P = 0.062$). Multiple regression analysis indicates that the VO_2max in mL.kg $LLM^{-1}.min^{-1}$ can be predicted from the levels of SOD2, total Nrf2 and Keap1 ($r = 0.58$, $P < 0.001$), with SOD2 being the main predictor explaining 28 % of the variance in VO_2max, while Nrf2 and Keap1 explained around 3 % of the variance each (Table 2). Sex explained 3.6 % of the variance in VO_2max.

3.6. Associations between $pSer^{40}$-Nrf2 and the Nrf2/Keap1 ratio and antioxidant enzymes

Significant associations were observed between $pSer^{40}$-Nrf2 and catalase ($r = 0.64$, $P < 0.001$), GR ($r = 0.33$, $P < 0.001$), and Keap1 ($r = -0.25$, $P < 0.001$). Likewise, significant associations were observed between Nrf2/Keap1 ratio and catalase ($r = 0.65$, $P < 0.001$) and GR ($r = 0.41$, $P < 0.001$). There was also an association between the percentage of MHC I + MHC IIa and the protein expression levels of GR ($r = 0.27$, $P = 0.025$, $n = 70$), catalase ($r = 0.24$, $P = 0.045$, $n = 70$), and SOD2 ($r = 0.24$, $P = 0.048$, $n = 69$).

4. Discussion

In the present investigation, the Nrf2/Keap1 and the Nrf2-regulated antioxidant enzymes (SOD1-2, catalase, GR) expression levels in skeletal muscle have been determined for the first time in a large sample of males and females with marked differences in adiposity, cardiorespiratory fitness, and age. The present findings show that Nrf2 and the main antioxidant enzymes in human skeletal muscle are expressed in equal amounts in males and females when sex differences in VO_2max, age and adiposity are accounted for. We have also demonstrated that a greater aerobic fitness (i.e., a higher VO_2max per kg LLM) associates with increased basal expression of total Nrf2, Keap1 and SOD2. Our findings also show that SOD2 is the strongest predictor of VO_2max in human skeletal muscle, while SOD1 protein expression increases with ageing after accounting for differences in VO_2max and body fat percentage. Finally, we have also shown that a BMI $\geq$ 25 kg m^{-2} is associated with increased levels of $pSer^{40}$-Nrf2, $pSer^{40}$-Nrf2/Total Nrf2 ratio and SOD1 protein expression levels, after accounting for differences in age and VO_2max normalized to the lean mass of the lower extremities.

4.1. Why are some antioxidant enzymes associated to VO_2max?

Superoxide (O_2^-) and H_2O_2 can be generated in at least 10 different mitochondrial sites during aerobic metabolism and at several extramitochondrial locations by nicotinamide adenine dinucleotide phosphate oxidases (NADPH Oxidases or NOXs), Xanthine Oxidase, Phospholipase A2 (PLA2), Lipoxygenases and Cyclooxygenases [43,44]. Although the relative importance of these sources of O_2^- and H_2O_2 during exercise has yet to be established, it is thought that at low and moderate exercise intensities most O_2^- and H_2O_2 have a mitochondrial origin, while at high exercise intensities, extramitochondrial sources may predominate [45]. In contrast to the general belief, the mitochondrial production of O_2^- and H_2O_2 is lower in conditions mimicking moderate or intense aerobic exercise than at rest [43]. Thus, performing low or moderate-intensity exercise should not require more antioxidant capacity than needed for resting conditions. However, experiments carried out in humans using electron paramagnetic resonance spectroscopy have detected an increase in biomarkers of oxidative stress in muscle biopsies [46], which should not have been detected had the ROS production remained below the resting values observed in isolated mitochondria tested in biochemical environments mimicking exercise conditions [43]. Thus, either mitochondrial O_2^- and H_2O_2 production is increased during aerobic exercise, or there is a large increase in extramitochondrial O_2^- and H_2O_2 production, as suggested by experiments showing reduced oxidative stress and blunted ROS-mediated signalling when exercise is performed after the administration of allopurinol, a xanthine oxidase inhibitor [47,48]. Increased O_2^- production during aerobic exercise is also supported by the fact that several studies have reported increased skeletal muscle SOD2 expression in endurance-trained athletes [49,50] and in response to endurance training [2,3,51,52]. However, other studies have reported no effects of training on antioxidant enzymes or glutathione status in skeletal muscle [53,54]. The level of muscle expression of SOD2 may also determine exercise capacity since heterozygous SOD2 gene–knockout mice, which have 30–80 % lower expression of SOD2, have reduced exercise capacity [55].

The present investigation has shown that SOD2, a mitochondrial antioxidant enzyme, is the antioxidant enzyme with the highest predictive value for VO_2max. This could indicate that an increased capacity to quench the free radicals produced during mitochondrial respiration is likely a critical factor for a higher VO_2max. This interpretation aligns well with the fact that animals with exceptional VO_2max possess remarkably increased levels of SOD in their skeletal muscles [56]. In agreement, it has been reported that conditional knockout of Mn-SOD (SOD2) targeted to type IIB skeletal muscle fibres in mice increases oxidative stress and is sufficient to alter muscle strength and aerobic exercise capacity [57]. In contrast, SOD2 overexpression reduces fibrosis and pro-apoptotic signalling in ageing hearts [58]. However, some studies have reported a lack of SOD increase with endurance [53,54,59] or sprint interval training [60] despite improvements in VO_2max. This divergence between studies could be explained by the variability of measurements combined with the small number of participants, the training programme, and the fitness levels of the participants in training studies.

4.2. Nrf2 is associated with VO_2max

The present investigation shows for the first time an association between Nrf2 total protein expression and VO_2max per kg of lower extremities lean mass. This manner of expressing VO_2max gives a better assessment of the intrinsic capacity of active muscles to uptake and utilize O_2. Thus, the observed association between VO_2max per kg of active muscle suggest that the local expression of Nrf2 is linked to changes in skeletal muscle that facilitate aerobic energy production. Accordingly, abrogation of Nrf2 expression reduces muscle performance [8], while the opposite is observed with Nrf2 overexpression in mice [6,61]. Increased levels of Nrf2 may improve endurance by increasing the antioxidant capacity [61], which, in turn, may counteract the pro-fatigue action of O_2^- and H_2O_2 during prolonged or fatiguing muscle contractions [62]. Besides, a sustained basal elevation of Nrf2 may promote mitochondrial biogenesis and enhance mitochondrial density [63,64]. Increased mitochondrial density is a pivotal feature of endurance-trained muscles and has been associated with increased O_2 extraction capacity and more capacity for fatty acid oxidation [65].

4.3. Age is associated with increased SOD1 protein expression in human skeletal muscle, regardless of VO_2max and body fat percentage

In most previous research with humans, markers of oxidative stress have been determined primarily in the circulation. Due to the necessity of muscle biopsies, skeletal muscle oxidative stress has been less studied in humans. Skeletal muscle oxidative stress has been reported with age [66–68], hypertension [69], and obesity in women [59] and men [2], and patients with chronic pulmonary obstructive disease (COPD) [70]. In these conditions, the oxidative stress is due to excessive production of RONS since the antioxidant mechanisms are unchanged or insufficiently increased to fully counteract the increased RONS production [5].

In the present cohort, a positive association between age and SOD1 protein expression has been observed, which remained present after accounting for the percentage of body fat and VO_2max. In agreement, a 92 % higher expression of SOD1 has been observed in m. *vastus lateralis* of ten physically active elderly males (mean age 71 years old) compared to ten physically active younger males (mean age 23 years old) [71]. The present finding confirms the association between ageing and SOD1 in a much larger cohort, including males and females. It is shown that this association is not explained by the reduction of VO_2max and the increase of the percentage of body fat with ageing. It has been proposed that the lifespan may be determined by the accumulation of oxidative damage to critical structures [72,73], among other factors [74]. The increase of SOD1 with ageing may reflect a counterregulatory response to balance an increased rate of O_2^- production [75], which seems necessary even after accounting for differences in VO_2max, as shown in the present research.

4.4. Being overweight or obese is associated with increased expression of $pSer^{40}$-Nrf2, its phosphorylation ratio and SOD1, while Keap1 protein expression is reduced

The present findings indicate that RONS production is likely elevated in obesity, which triggers adaptative mechanisms mediated by the activation of Nrf2 signalling, facilitated by its higher phosphorylation level and the reduction of its inhibitory factor Keap1. This results in an increased expression of SOD1, which is the most abundant antioxidant enzyme in skeletal muscle [5].

In agreement with our findings, increased expression of SOD1 has been reported in 12 middle-aged women [59] and 9 obese men [2] compared to lean peers. Also, in concordance with the present results, no elevation of SOD2 or catalase was seen in obese patients in these two previous studies [2,59]. After a 12-week endurance training programme that improved VO_2max (expressed normalized to the whole-body lean mass), SOD1 protein expression was normalized in the female participants, despite no change in body fat percentage [59]. In contrast, after 12-week endurance training, SOD1 remained elevated in the obese males, despite improvements in VO_2max and no change in the percentage of body fat [2]. Superoxide dismutase 2 was increased after 12 weeks of endurance training in males [2] but not in females [59]. In the present investigation, VO_2max was related to SOD2 protein expression in males and females ($r > 0.50$).

The reason why Nrf2 signalling and the antioxidant enzymes are increased in the *vastus lateralis* with obesity, even after accounting for the confounding effects of age and the percentage of body fat, remains unknown. The most plausible explanation for enhanced Nrf2 signalling in the skeletal muscle of patients with obesity is the low-grade chronic inflammation that develops with obesity, which by multiple mechanisms may cause oxidative stress [76]. In favour of this explanation, several studies have reported higher levels of oxidative damage markers (especially protein carbonyls and 4-hydroxy-2-nonenal, HNE adducts) in the skeletal muscle of obese patients compared to lean counterparts [2,59]. It could also be that the mechanical overload caused by obesity explains a similar response to that described in subjects doing strength training [77]. Another mechanism could be the activation of Nrf2 signalling mediated by NFκB, which in turn responds to pro-inflammatory cytokines increased in the circulation in obese patients [78].

4.5. Sex differences in skeletal muscle antioxidant enzymes, Keap1 and Nrf2

In the present investigation, no significant differences in the protein expression levels of Nrf2, Keap1 and antioxidant enzymes were observed after accounting for age, body composition and VO_2max differences. Only one previous study has assessed the antioxidant enzyme status in human skeletal muscle biopsies obtained from males and females undergoing orthopaedic surgery (57 males and 63 females, aged from 17 to 91 years old) [79]. In agreement with our results, no sex differences were observed in SOD1, SOD2, catalase and glutathione peroxidase activities in muscle biopsies obtained from the *rectus abdominis*, *vastus lateralis* and *gluteus maximus* [79]. The collection conditions and time to freezing are not reported, nor are the specific characteristics of the subjects (underlying disease, medications, body composition, and physical activity/fitness), which limits the interpretation of the results. Barreiro et al. did not find significant sex differences in SOD2 or catalase in healthy volunteers' *external intercostalis* and *vastus lateralis* (12 males and 12 females) [67].

4.6. Limitations

This study has focused on assessing the changes in the protein expression levels of the main antioxidant enzymes and their main regulatory factors, i.e., Keap1 and Nrf2. Thus, future studies should also evaluate enzymatic activities and the ratio of total enzyme protein expression to enzymatic activity to check for potential enzyme deactivation, which should be minimal at rest in a healthy muscle. Although we have taken care to account for differences in physical fitness, body composition and age as confounding factors, we did not control for the diet. A diet rich in natural polyphenols may have anti-inflammatory and antioxidant effects [80,81]. Although daily physical activity was not measured, we determined VO_2max directly and normalized its value to the lower extremities lean mass, which allows a direct comparison between males and females or participants with different ages and levels of physical activity since muscle mass and VO_2max are primary outcome variables modified by physical activity. Although all females were premenopausal, they were assessed disregarding the potential influence of the menstrual cycle phase. In plasma, SOD and catalase activities are lower in the luteal than the follicular phase of the menstrual cycle [82]. So far, no study has determined whether skeletal muscle antioxidant enzymes fluctuate with the menstrual cycle in women. Although the most abundant antioxidant enzymes in skeletal muscle were measured [83], other antioxidant enzymes such as glutathione peroxidase, peroxiredoxins, thioredoxins, thioredoxin reductases, and glutaredoxins were not determined. Finally, the present results do not exclude potential differences in non-enzymatic antioxidants, which were not assessed.

In summary, this is the first study demonstrating an association between VO_2max per kg of lean mass of the lower extremities and the expression of some antioxidant enzymes in human skeletal muscle. We have shown that, among the antioxidant enzymes in human skeletal muscle, SOD2 protein expression is the stronger predictor of VO_2max and that SOD1 protein expression increases with ageing after accounting for differences in VO_2max and percentage of body fat. Moreover, our current observations indicate that males and premenopausal females have similar protein expression levels of Nrf2 and antioxidant enzymes in human skeletal muscle. Finally, this investigation shows that overweight and obesity are associated with elevated protein expression levels of $pSer^{40}$-Nrf2, $pSer^{40}$-Nrf2/Total Nrf2 ratio and SOD1, after accounting for differences in age and VO_2max. Overall, at the group level, higher aerobic fitness is associated with elevated basal expression of certain muscle antioxidant enzymes, which may explain some of the benefits of regular exercise.

Disclosure summary

The authors have nothing to disclose.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2023.10.393.

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ESTUDIO 3

SCANDINAVIAN JOURNAL OF MEDICINE & SCIENCE IN SPORTS

Physiological and molecular predictors of cycling sprint performance

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Physiological and molecular predictors of cycling sprint performance

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Running title: Muscle phenotypic determinants of sprint performance

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DATA AVAILABILITY

Deidentified participant data are available from the senior author (ORCID: 0000-0002-9215-6234) on reasonable request for research purposes.

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CONFLICT OF INTEREST DISCLOUSURE

The authors declare that they have no conflict of interest.

Ethics approval statement

All experiments were performed per the Declaration of Helsinki after ethical approval (CEIH-2017-13).

Abstract

The study aimed to identify novel muscle phenotypic factors that could determine sprint performance using linear regression models including the lean mass of the lower extremities (LLM), myosin heavy chain composition (MHC), and proteins and enzymes implicated in glycolytic and aerobic energy generation (citrate synthase, OXPHOS proteins), oxygen transport and diffusion (myoglobin), ROS sensing (Nrf2/Keap1), antioxidant enzymes, and proteins implicated in calcium handling. For this purpose, body composition (dual-energy X-ray absorptiometry) and sprint performance (isokinetic 30-s Wingate test: peak and mean power output, Wpeaki and Wmean) were measured in young physically active adults (51 males and 10 females), from which a resting muscle biopsy was obtained from the musculus *vastus lateralis*. Although females had a higher percentage of MHC I, SERCA2, pSer^{16}/Thr^{17}-phospholamban, and Calsequestrin 2 protein expressions (all $P<0.05$), and 18.4% lower phosphofructokinase 1 protein expression than males ($P<0.05$), both sexes had similar sprint performance when it was normalized to body weight or LLM. Multiple regression analysis showed that Wpeaki could be predicted from LLM, SDHB, Keap1 and MHC II % ($R^2=0.62$, $P<0.001$), each variable contributing to explain 46.4, 6.3, 4.4 and 4.3 % of the variance in Wpeaki, respectively. LLM and MHC II explained 67.5% and 2.1% of the variance in Wmean, respectively ($R^2=0.70$, $P<0.001$). The present investigation shows that SDHB and Keap1, in addition to MHC II %, are relevant determinants of peak power output during sprinting.

1 INTRODUCTION

Previous studies have reported an association between thigh muscle volume [1,2], lower extremities lean mass [3,4] and lower extremities volume [5] and the peak power output reached during maximal sprinting on the cycle ergometer. However, muscle mass leaves a large proportion of the variability in sprint performance unexplained, meaning that other factors should play a role. In this regard, a higher percentage of myosin heavy chain type II (MHC II) has been associated with faster contraction speeds and power generation *in vitro* and *in vivo* generation [5-9], while the relative area of the muscle occupied by type II fibres has been associated with a higher mean power output during the Wingate test [10]. However, very few attempts have been made to quantify the role played by potential predictive variables beyond the effect of muscle mass and myosin heavy chain composition [5 1]. Even with the inclusion of muscle volume and MHC II, a large portion of the variance in peak power output remains unexplained [1,5], implying that more muscle phenotypic features should be implicated.

In theory, the peak power output may depend on muscle features allowing the achievement of higher peak force or muscle shortening speed during the sprint. Since peak power output depends on the optimal combination of force and muscle contraction velocity, the proteins implicated in Ca^{2+} handling and Ca^{2+} sensitivity could be determinants of peak and mean power output [11]. In addition, all processes implicated in energy metabolism could affect peak and mean power output. During the Wingate test, 20-30 % of the energy is provided by oxidative phosphorylation while the rest depends on substrate-level phosphorylation [12-14], with a greater contribution by glycolysis than phosphagens (ATP and phosphocreatine) [13-15].

During sprint exercise, substantial amounts of reactive oxygen and nitrogen species (RONS) are produced, which may be deleterious for sprint performance [16,17] unless timely counteracted by redox-regulating proteins and enzymes like nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and kelch-like ECH-associated protein 1 (Keap1). Keap1 is a cysteine-rich protein that acts as an oxidative and electrophilic stress sensor. Under resting unstressed conditions, most Keap1 is bound to Nrf2, preventing Nrf2 translocation to the nucleus. Keap1 is an adaptor protein for a Cul3/Rbx1 E3 ubiquitin ligase complex, which ubiquitinates Nrf2 for proteasomal degradation. However, in the presence of oxidative or electrophilic stress, several Keap1 cysteine residues undergo covalent modifications leading to Keap1 detachment from Nrf2 [18-20]. Free Nrf2 can then translocate to the nucleus, where it binds to antioxidant response elements (AREs) to activate the expression of genes involved in the antioxidant response and mitochondrial biogenesis [21,22].

Therefore, this research aimed to determine in male and female adults which muscle phenotypic factors could determine sprint performance using a linear regression model assessing proteins and enzymes implicated in glycolytic and aerobic energy generation (citrate synthase, OXPHOS proteins), oxygen transport and diffusion (myoglobin), reactive oxygen and nitrogen species (RONS) sensing (Nrf2/Keap1), antioxidant enzymes, and proteins implicated in calcium handling.

The central hypothesis is that, given the role attributed to RONS in fatigue during high-intensity exercise [16,17], muscle molecular components enhancing redox regulation, like Nrf2/Keap1 and antioxidant enzymes regulated by Nrf2 could also be determinants of sprint performance. We also hypothesized that higher expression of enzymes implicated in Ca^{2+} handling (Serca1, Serca2, and Phospholamban, PLB),

glycolytic energy production (Phosphofructokinase 1, PFKM), O_2 diffusion (Myoglobin) and O_2 utilization (Citrate synthase, oxidative phosphorylation proteins) will be associated with increased sprint performance.

2 METHODS

2.1 Subjects

Fifty-one males and ten females participating in research projects carried out in our laboratory to determine mechanisms of fatigue during high-intensity exercise [23-25], were included in this study (Table 1). To be eligible for participation in this research, volunteers had to be physically active and healthy, body mass index above 18 and below 30, without contraindications for maximal exercise, be non-smokers and not taking any drug or medication. All participants volunteered to participate in the corresponding studies and signed written informed consent after receiving complete information regarding the aims of the studies and potential side effects of the procedures. Gender and sex identification were self-disclosed during the recruitment phase. All respondents identified themselves as cis-gender. All female participants were eumenorrheic, not on oral contraceptives, and were assessed randomly during their menstrual cycle phases [26]. This methodology aligns with studies indicating consistent sprint and high-intensity performance outcomes across menstrual phases [27-29]. All experiments were performed per the Declaration of Helsinki after ethical approval (CEIH-2017-13).

2.2 Main procedures

Participants were instructed to refrain from engaging in intense physical activity 48 hours prior to all laboratory evaluations, including the muscle biopsy. Additionally,

they were advised to avoid sparkling beverages, caffeine, and alcohol for 24 hours before all tests. Throughout the study, no dietary supplements or vitamins were allowed.

The study included, on separate visits to the laboratory, a pre-testing and familiarization phase, followed by assessing their VO_2max by an incremental test to exhaustion with verification and performance of an isokinetic 30s Wingate test. The pre-testing and familiarization phase included the subjects reported to the laboratory early in the morning, following a 12-h overnight fast for assessment of their body composition using dual-energy x-ray absorptiometry (Lunar iDXA, GE Healthcare, Milwaukee, WI, USA). The anatomical regions of interest were delineated manually from the whole-body scans, as previously reported [30]. On subsequent visits to the laboratory, the volunteers performed an incremental exercise to exhaustion and two isokinetic Wingate tests at 80 rpm for familiarization purposes. Adjustments to the seat and handlebar were carefully customized to the subject´s anthropometric measurements during the first visit and were kept consistent throughout the following visits. Special care was taken into familiarize the participants with the performance of sprinting in isokinetic mode at 80 rpm while remaining seated in the saddle. Exercise tests took place in an air-conditioned laboratory with an ambient temperature of ~21°C, a relative humidity of 60–80%, and ~735 mmHg atmospheric pressure.

2.3 Assessment of VO_2max

The VO_2max, maximal power output (Wmax) and maximal hear rate (HRmax) were determined in normoxia (F_IO_2: 0.21, P_IO_2: 144 mmHg) using an incremental exercise test until exhaustion, including a verification phase [31]. The incremental exercise was

performed in a post-absorptive state, at least 4 hours after a light meal using a cycle ergometer (Lode BV, Groningen, The Netherlands). In forty subjects (30M/10W), the test began with a 3-minute stage at 20 W, which was subsequently increased every three min by 15 W for females and 20 W for males until the respiratory exchange ratio (RER) reached or exceeded 1.00. Following this, the intensity was raised every minute by 10 W in females and 15 W in males until exhaustion. In the remaining 21 male subjects, the initial load was set at 80 W, which was increased by 30 W every 2 min until exhaustion. The peak intensity achieved was denoted as the Wmax. Upon exhaustion, the ergometer was unloaded, and the subjects continued pedalling at a low cadence (30-40 rpm) for 3 minutes to facilitate recovery. Right after, the verification phase began at Wmax + 5 W for a minute, increased every 20 s by 4 W in females and 5 W in males, continuing until participants could no longer sustain the effort. Exhaustion was defined either by the inability to sustain a pedalling rate of more than 50 rpm for 5 seconds or by an abrupt cessation of pedalling. Verbal encouragement was provided throughout the last phases of every test.

During the incremental exercise test, the VO_2 was measured breath-by-breath by indirect calorimetry (Vyntus, Jaeger-CareFusion, Höchberg, Germany), which has been validated by a butane combustion test [32]. The metabolic cart was calibrated immediately before each with room air (20.93% O_2 and 0.05% CO_2) and calibration gases purchased from the manufacturer (16% O_2 and 5% CO_2). The flowmeter was calibrated for low (0.2 L/s) and high (2 L/s) ventilation flows immediately before each test. Breath-by-breath data were averaged every 20 seconds, and the highest 20-s averaged VO_2 achieved during either the incremental test or the verification phase was

taken as the VO_2max [33]. Heart rate (HR) was registered every 1 s (RS400 and RS800, Polar Electro, Woodbury, NY, USA).

2.4 Wingate test

The Wingate test consisted of a 30-s all-out sprint performed on a separate testing day, at least 4 hours after a light meal (Lode Excalibur Sport 925900, Groningen, The Netherlands). The Wingate test was preceded by a standardized warm-up consisting of 1 min of unloaded pedalling, 2 min at 40 W or 60 W, 3 min at 60 or 80 W, 1 min at 80 or 100 W, 1 min at 100 or 120 W, and 1 min at 120 or 140 W for females and males, respectively. At the end of the warm-up, the participants recovered by pedalling at a low cadence (20-40 rpm) for 5 min with the ergometer unloaded. The Wingate test was performed in isokinetic mode at a fixed cadence of 80 (41 males and 10 females) or 100 rpm (10 males), with the participants remaining on the saddle at all times and while strong verbal encouragement was given to ensure that a maximal effort was provided. Prior to each sprint, participants accelerated the flywheel near to the prescribed cadence and were instructed to pedal below that cadence until the 5-second countdown started, while reminded to sprint as hard as possible from the start to the end of the sprint and to refrain from a premature start after the start signal is given. Utilizing the ergometer configured in isokinetic mode, the resistance presented is continuously adjusted through an automated servocontrol mechanism. This apparatus exclusively permits a consistent pedalling cadence of 80 (±1) rpm, ensuring that any diminution in the force applied to the pedals leads to a corresponding reduction in the ergometer's resistance, and conversely, an augmentation in force results in an increase in resistance. All participants, regardless of the sport cycling

footwear chosen, were tightly attached to the pedals using additional fastening straps. Instantaneous power values were obtained using Lode Ergometry Manager Software (LEM; Lode BV, Groningen, The Netherlands) from which instantaneous peak power output (Wpeaki) was obtained, while the peak power output in 1-s averages (Wpeak1-s) and the mean power output (Wmean) were calculated.

2.5 Muscle biopsies

Vastus lateralis muscle biopsies were performed in the morning after a 12-h overnight fast from the middle third of one of the two thighs, using Bergstrom's needles and applying suction, as previously reported [23]. The leg to be biopsied was assigned randomly, and the skin and subcutaneous tissue were infiltrated with 2% lidocaine (1-2 mL), taking special care to avoid anaesthetic injection below the fascia. Ten minutes later, a 5 mm incision was performed, and the needle was introduced with a 45° inclination towards the foot to position the biopsy window of the needle about 2 cm below the fascia. All biopsies were immediately frozen in liquid nitrogen and store at -80°C until analysed.

Protein extraction and western blotting

Muscle lysates preparation was carried out as previously reported [34]. Briefly, lysates protein concentration was determined by the bicinchoninic acid assay [35]. Then, approximately 10 mg of muscle were homogenized in urea lysis buffer (6 M urea, 1% SDS), 50X Complete protease inhibitor and 10X PhosStop phosphatase inhibitor cocktails (Roche). This mixture was centrifuged for 12 min at 25,200 g at 16 °C and the supernatant was diluted with electrophoresis loading buffer (160 mM Tris-HCl, pH 6.8, 5.9% SDS, 25.5% glycerol, 15% β-mercaptoethanol- bromophenol blue). All proteins

were assayed at optimal amounts, i.e., in the linear portion of the antigen-antibody reaction, as revealed by optical density response for protein amounts ranging from 1 to 35 µg. Equal amounts of protein of each sample (1.5-15 µg) were loaded for SDS-PAGE using the system of Laemmli [36], followed by transference onto the polyvinylidene fluoride (PVDF) membranes for protein blotting (Bio-Rad Laboratories, Hercules, CA, USA). To reduce the variability between gels, test samples were run with an equal protein amount from an internal human control sample (non-experimental and same as during linearity optimization) loaded in triplicate or quadruplicate. The densitometric value of the protein of interest was normalized to the mean value of the internal control sample included onto the gel.

Membrane blocking was achieved by incubation for 1h in 4% bovine serum albumin or 5% non-fat dried milk powder (blotting-grade blocker) diluted in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) (BSA or Blotto blocking buffer) and incubated overnight at 4°C with primary antibodies. Afterwards, the membranes were washed and incubated for 1 hour at room temperature with an HRP-conjugated anti-rabbit or anti-mouse antibody (diluted 1:5000 to 1:20000 in 5% Blotto blocking buffer in all instances) followed by chemiluminescent visualization with Clarity™ Western ECL Substrate (Bio-Rad Laboratories) with the ChemiDoc™ Touch Imaging System (Bio-Rad Laboratories). Band density was determined using the Image Lab© software 5.2.1 (Bio-Rad Laboratories). Reactive Brown 10 (Sigma-Aldrich, St. Louis, MO, USA) staining was used to control for equal loading and transfer efficiency. The corresponding catalogue numbers of primary antibodies were as follows: anti-SOD1 (no. ab16831), anti-pSer^{40} Nrf2 (no. ab76026), anti-Nrf2 (no. ab62352), and the anti-OXPHOS premixed cocktail

antibody (total OXPHOS human antibody cocktail, no. ab110411) which includes antibodies against Complex I subunit NADH dehydrogenase [ubiquinone] 1 beta subcomplex (NDUFB8, ab110242), Complex II subunit succinate dehydrogenase [ubiquinone] iron-sulfur (SDHB, ab14714), Complex III subunit cytochrome b-c1 complex subunit 2 (UQCRC2, ab14745), Complex IV subunit cytochrome c oxidase subunit 2 (COXII, ab110258), and ATP synthase subunit alpha (ATP5A, ab14748) were purchased from Abcam (Cambridge, UK). Anti-Myoglobin (no.25919), anti-SOD2 (no. 13141), anti-pSer^{16}/Thr^{17} Phospholamban (no. 8496) and anti-Catalase (no. 14097) were obtained from Cell Signalling Technology (Danvers, MA, USA). Anti-Calsequestrin1 (CSQ1) (no. C0618), anti-Calsequestrin2 (CSQ2), (no. 3868), anti-SERCA1 (no. WH0000487M1), and anti-SERCA2 (no. S1439) were ordered from Sigma-Aldrich. Anti-Citrate synthase (16131-1-AP), anti-Keap1 (no. 10503-2-AP), anti-Phosphofructokinase 1 (PFKM) (55028-1-AP) and Glutathione reductase (GR) (18257-1-AP) were acquired from Proteintech (Rosemont, IL, USA). Secondary HRP-conjugated goat anti-mouse (no.115-035-003) and goat anti-rabbit (no. 111-035-144) antibodies were purchased from Jackson ImmunoResearch Inc. (West Grove, PA, USA). See Supplementary Table 1 for complementary information.

Myosin heavy chain analysis

Myosin heavy chain isoform composition analysis was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), as previously reported [25]. Briefly, 7.5-10 µg of the same western blot-ready protein extracts used for muscle signalling were loaded in triplicate onto the same gel, together with two internal control samples of known MHC composition (used for quality control). SDS-PAGE gels

containing a 3% acrylamide (v/v) phase (stacking gel) were run at 4 °C for ~12 h at 70 V and afterwards on a 6% acrylamide (v/v) and 30% glycerol (v/v) phase (resolving gel) for ~20 h at 350 V. Bands were identified by staining with Coomassie Blue for ~1 hour, subsequently distained with a 40% methanol (v/v) and 10% glacial acetic acid (v/v) solution for ~ 1 hour and finally submerged in distilled water ~1 hour to optimize background subtraction. The MHC composition was determined by scanning the gel with a densitometry scanner (GS-800 Imaging Densitometer, Bio-Rad Laboratories, Hercules, CA, USA) followed by quantification (Image Lab© software 5.2.1, Bio-Rad Laboratories).

Statistics

The normal distribution of variables was verified using the Shapiro-Wilks test, and variables that were not normally distributed were logarithmically transformed before further analysis. Descriptive data are reported as the mean ± standard deviation (SD). Sex differences were analysed using a two-tailed unpaired t-test, with appropriate correction in case of failure to pass Levene's test for equality of variances. Linear relationships between variables were determined using simple and multiple linear regression analyses, including sex as a categorical variable in the prediction models. Statistical significance was set at $P<0.05$. Statistical analyses were performed using IBM SPSS Statistics v.21 for Mac (SPSS Inc., Chicago, IL, USA) and Jamovi v1.8.1. (Jamovi project, 2021).

3 RESULTS

3.1 Physical characteristics and performance

The physical characteristics of the participants are reported in Table 1. Females had lower height, body mass and whole-body lean mass (LM) than males and a higher body fat percentage. When VO_2max was expressed in absolute values, normalized to whole body mass and normalized to lean mass of the lower extremities (LLM), values were 34.2, 16.1, and 8.6% lower in females than males, respectively. Males had superior sprint performance than females in absolute values, although those sex differences disappeared after normalization to body weight, LM and LLM (Table 2).

3.2 Sex differences in skeletal muscle phenotype

Females had a higher percentage of MHC I than males, while males had a higher percentage of MHC IIa than females (Table 1). No between-sex differences in MHC IIx were observed (Table 1). Keap1, Nrf2, and the antioxidant proteins assessed had similar levels of expressions in males and females (Fig. 1). The OXPHOS mitochondrial proteins were expressed similarly in males and females (Fig. 1). Citrate synthase and myoglobin were also similarly expressed in both sexes. Phosphofructokinase protein expression was 18.4% lower in females than in males (P=0.049). Marked sex differences were observed in Ca^{2+} handling proteins (Fig. 1), where SERCA2, $pSer^{16}/Thr^{17}$ PLB (26 kDa + 12 kDa isoforms), and CSQ2 protein expressions were 2.8 and 1.7, 1.7-fold higher in females than males, respectively (P<0.05). Representative immunoblots are depicted in Fig. 2.

3.3 Relationships between sprint performance and skeletal muscle phenotype

3.3.1 Myosin heavy chain composition

In the whole group of subjects, a higher percentage of MHC II was associated with greater Wpeaki and Wmean per body mass (both r=0.32, P<0.05). This association was

attenuated when expressed per kg of LM (r=0.26, P=0.041; r=0.27, P=0.033, respectively). However, when expressed per kg of LLM, the correlation did not reach statistical significance (r=0.24, P=0.06). No significant associations were observed between Wingate test performance and MHC IIx or MHC I+IIa.

3.3.2 The lean mass of the lower extremities is the main determinant of sprint performance.

There was a linear relationship between Wpeaki or Wmean and the LM (r=0.60 and r=0.76, respectively, both P<0.001) and the LLM (r=0.68, r=0.82, respectively, both P<0.001), indicating a higher predictive value for LLM. This relationship was similar in males and females, with no sex interaction. Since there is collinearity between LM and LLM (r=0.91, P<0.001), LLM was retained for multiple regression models to predict sprint performance.

Multiple regression analysis showed that Wpeaki could be predicted from LLM, SDHB, Keap1 and MHC II % (R^2=0.62, P<0.001), each variable contributing to explain 46.4, 6.3, 4.4 and 4.3 % of the variance in Wpeaki, respectively (Table 3). Multiple regression analysis showed that the main variables contributing to explain the variance of Wmean were LLM and MHC II % (R^2=0.70, P<0.001), which explained 67.5 and 2.1% of the variance in Wmean, respectively (Table 4). None of the other phenotypic variables examined made any significant contribution to enhance the predictive value of LLM and MHC II %. Sex did not significantly enhance the predictive value of these two models (Tables 3 and 4).

In females, there was an association between Wpeaki normalized to LLM and PFKM protein expression (r=0.81, P=0.005, n=10) and Log MHC II % (r=0.69, P=0.027, n=10). Similar associations were observed between Wmean and PFKM protein

expression (r=0.67, P=0.033, n=10), Log MHC II %(r=0.82, P=0.004, n=10), and SERCA1 protein expression (r=0.70, P=0.024, n=10). In males, Wpeaki normalized to LLM was associated with NDUFB8 protein expression (r=-0.29, P=0.0462 n=51), whilst Wpeaki normalized to LLM was associated with the ratio $pSer^{40}$ Nrf2/Nrf2 total (r=-0.29, P=0.046 n=51), and Log MHC II % (r=0.31, P=0.029, n=51). Log MHC II % did not correlate with SDHB (r=-0.17, P=0.23, n=51) nor Keap1 (r=0.09, P=0.55, n=51).

4 DISCUSSION

The present investigation shows that the main two variables determining sprint performance during exercise on the cycle ergometer are the active muscle mass and the percentage of type II fibres, as shown by the peak and the mean power output developed during a 30-s sprint. The main novelty of the present investigation is that, despite the large number of phenotypic variables examined, which included proteins and enzymes implicated in glycolytic and aerobic energy generation (PFKM, citrate synthase, OXPHOS proteins), oxygen transport and diffusion (myoglobin), RONS sensing (Nrf2/Keap1), antioxidant enzymes, and proteins implicated in Ca^{2+} handling, only SDHB and Keap1 were significantly associated with sprint performance. Interestingly, once LLM was considered, no significant sex differences were observed in sprint performance, further emphasizing the dominant role of the active muscle mass for peak and mean power output in a 30-s sprint on the cycle ergometer.

4.1 The active muscle mass is the main determinant of sprint performance

In agreement with our results, Kordi et al. [2] reported a significant correlation between quadriceps and hamstring muscle volume, as assessed by magnetic resonance imaging, and peak power output in 35 male cyclists specialized in sprint and endurance

modalities, most of them elite athletes. Kordi et al., like us, used isokinetic tests, although their subjects performed repeated 4-s sprints with a fly start at different cadences to determine the optimal cadence, which ranged between 112-162 rpm, with a mean value of 131 rpm. This allowed their cyclists to achieve an average peak power output of 1260 W, with a range between 775-2025 W. Also in line with our results, it has been reported in 28 male track and road cyclists that *vastus lateralis* muscle volume assessed by 3D ultrasonography explained 38% of the variance in peak power output and that including MHC II in the regression model improved the predictive capacity of the model to 65% [1]. The lower predictive value of *vastus lateralis* volume in van der Zwaard et al. [1] could potentially be attributed to the involvement of a larger number of muscles during sprint cycling [37].

Despite the fact that in the present investigation the participants were not cyclists and performed the test from a stopped start, the mean Wpeaki value reached by our male participants was just 22% lower than achieved by the sprint cyclists studied by Kordi et al. [2]. Although our subjects would be expected to reach Wpeaki at an optimal cadence close to 100-110 rpms [38 39], the difference in Wpeaki between 80 and 100 rpms is below 4% and between 80 and 120 rpm is 1.6% in elite cyclists [40], meaning that setting the cadence at 80 rpm for most of our subjects, should have barely affected the interpretation of our results [41]. Other factors, such as differences in training background, lean body mass, and the type of cycle ergometer, could explain our subject's lower mean power output compared to those reported in elite cyclists [2,40].

Conceptually, peak power output requires an optimal combination of force and muscle contraction velocity [42], represented by the muscle size and the percentage of

fast-twitch fibres. Our regression model attributes a markedly larger impact to the muscle mass, represented by the lean mass of the lower extremities. This is explained by the fact that the muscle cross-sectional area (or volume) and maximal isometric force are closely related, as well as cross-sectional area and muscle volume (or mass) in males, females, adults, elderly and children [43-48]. The speed of muscle contraction is determined by myosin heavy chain composition [49,50]. Individual muscle fibres tested *in vitro* show remarkably different maximal contraction speeds [51], with the fibres expressing MHC II being the fastest [49]. In agreement, the present results indicate that myosin heavy chain composition contributes to explaining differences in Wpeaki in humans, but its relative contribution is small (2%) compared to that of the muscle mass. This finding should, however, be interpreted cautiously because the tests were performed under isokinetic conditions at a fixed pedalling rate of 80 rpm, which may be more favourable for type 1 than type 2 fibres in humans [5,42,52]. However, our results match the Wpeaki measured in our laboratory 15 years ago using isoinertial Wingate tests in subjects of similar characteristics, which was 50.4 and 50.5 W·kg LLM^{-1} in 123 males and 32 females, respectively [4].

Previous studies have also reported a bivariate correlation between lean mass and mean power output during Wingate tests [3,4]. However, none of the previous studies carried out a comprehensive assessment of additional muscle phenotypic characteristics that could contribute to explain the human variability in mean and peak power output during sprint exercise. In the present investigation, we show for the first time that SDHB and Keap1, in addition to MHC II percentage, are relevant determinants of peak power output, as analysed in the following sections.

4.2. A high percentage of MHC II is positively associated with peak and mean power output during sprint exercise

A higher percentage of MHC II, which predominates in fast-twitch muscle fibres (FT), allows for faster shortening speed and greater peak power generation [5-9] due to the higher ATPase activity of MHC IIa and IIx compared to MHC I [50,53]. Although FT fibres are less efficient than slow-twitch (ST) fibres [54], under rested unfatigued conditions when ATP availability and resynthesis rate are not limiting, a higher MHC II percentage contributes to determining peak power and mean power output. However, the present investigation indicates that a larger muscle mass is more critical than a higher proportion of MHC II. This is supported by the similar peak and mean power output normalized to the LLM in males and females despite the higher expression of MHC II in the former. Besides, muscle maximal shortening speed is barely changed with training, meaning that the improvements in sprint performance elicited by training rely mainly on ameliorating muscle force [9]. Similarly, muscle force has been identified as the main factor explaining differences in sprint performance between young and elderly subjects [55,56].

4.3 The protein expression level of the mitochondrial enzyme SDHB in skeletal muscle is positively associated with peak power output

Mitochondrial complex II (succinate-ubiquinone oxidoreductase [SDH]) is formed by four subunits encoded in the nuclear genome (SDHA, SDHB, SDHC, and SDHD). Genetic suppression of the SDHB subunit is associated with increased basal cytosolic oxidant stress and has been shown to facilitate mitochondrial ROS production [57]. A similar mechanism may operate in human skeletal muscle, where a lower expression level of

SDHB may facilitate ROS production already in basal conditions and even more during exercise. On the other hand, a higher expression of SDHB may attenuate succinate accumulation [57], and so more during high-intensity exercise [58]. Succinate accumulation has been shown to elicit mitochondrial ROS production [59,60]. More recently, it has been shown that increased expression of SDHA leads to the accumulation of fumarate [61], which evokes succinylation of Keap1 in cysteines C151 and C288, resulting in disruption of the interaction between Keap1 and Nrf2, promoting Nrf2 signalling [62,63].

Thus, the fact that SDHB expression is positively associated with peak power output may indicate that a higher capacity of skeletal muscle to quench free radicals, oxidants and electrophiles during high-intensity exercise may be crucial for maximizing power output during sprint exercise. In agreement with this hypothesis, it has been shown that ROS can contribute to fatigue by reducing sarcoplasmic Ca^{2+} release and troponin Ca^{2+} sensitivity, among other potential mechanisms [11,64,65].

Although SDHB is an enzyme involved in oxidative phosphorylation and, therefore, intervenes in establishing the rate of aerobic ATP resynthesis, aerobic energy supply does not limit peak power output during sprint exercise in humans [15,66,67].

4.4 Why do subjects with increased peak power output have lower Keap1 resting levels?

The present study demonstrates for the first time that the basal expression level of Keap1 protein in human skeletal muscle is a negative predictor of peak power output. Previous research in transgenic mice unable to express Keap1 in their skeletal muscles has shown that Keap1 ablation in skeletal muscle is associated with enhanced

endurance performance, although this effect was observed only in female rodents [68]. These animals possess constitutively elevated Nrf2 protein levels, which promote phenotypic changes in muscle fibres, facilitating aerobic exercise [68]. Sprint performance relies primarily on substrate-level phosphorylation, which has two main components: the phosphagen and glycolytic metabolic pathways. Peak power output requires a fast rate of ATP resynthesis from phosphocreatine and glycolysis, both being higher in FT than ST fibres [13,69]. Genetic ablation of Keap1 in skeletal muscle is associated with enhanced expression of antioxidant enzymes, which could allow a more efficient counteraction of RONS produced in response to the extremely high glycolytic rates attained during the first seconds of a maximal sprint in human skeletal muscle [13,15,24,70].

Keap1 cysteines may be covalently modified by several endogenous metabolites [62,63,71,72]. For example, by methylglyoxal, an electrophile that results from the elimination of triose phosphate, which accumulates in conditions with high glycolytic flux, as occurs during sprint in normoxia [13,70] and sprint [15] or moderate-intensity exercise in hypoxia [73]. Methylglyoxal is highly reactive and could non-enzymatically cross-link and dimerize Keap1 molecules via a methyl imidazole-based linkage between cysteine (C151) and arginine (R15 or R135) residues, facilitating Nrf2 signalling [71]. More recently, it has been shown that accumulation of glyceraldehyde 3-p elicits S-lactoylation of several cysteines in Keap1 by mechanisms that have yet to be elucidated [72]. Since glyceraldehyde 3-p readily accumulates during sprint exercise [13], it could contribute to Nrf2 signalling by impeding the inhibitory action of Keap1 through its S-lactoylation.

Although a definitive answer to the question cannot be provided, low levels of Keap1 may permit higher expression of antioxidant and pentose phosphate pathway proteins [72], which prevent the negative effects of oxidants and electrophiles produced during the high glycolytic rates associated with maximal sprint performance [13,70]. Moreover, with lower levels of Keap1, more newly synthesized Nrf2 will escape inhibition by a less abundant Keap1, facilitating the Nrf2 response to acute exercise [74]. Additionally, reduced basal levels of Keap1 could also facilitate the adaptative response to training.

4.5 Perspectives

The lower levels of Keap1 at the start of exercise should facilitate Nrf2 activation, which elicits adaptations in skeletal muscle, preventing oxidative and electrophilic damage and accumulation of potentially toxic glycolytic intermediaries like methylglyoxal in subsequent exercises demanding high glycolytic rates. Thus, keeping low levels of Keap1 may be necessary for a physiological adaptation to sprint (or high-intensity exercise) in humans. Previous studies show that Keap1 protein is reduced in human skeletal muscle immediately after high-intensity exercise [74], although Keap1 pre-exercise levels are recovered shortly after exercise [74]. It remains to be elucidated whether sprint exercise training reduces resting Keap1 protein expression level in human skeletal muscle. Additional experiments are required to determine whether Keap1 dimerization and Keap1 S-lactoylation occurs during sprint exercise in humans and how the production of these Keap1 derivatives is modified by training, ageing, disease and if they display sexual dimorphism in humans.

Additional experiments manipulating the expression of SDHB would be required to clarify how changes in the expression of this protein could influence sprint performance.

In summary, we have identified novel muscle phenotypic characteristics associated with sprint performance in humans, which complement the two key variables in sprint performance, the active muscle mass and the percentage of type II fibres. The present findings provide further evidence for the role of Keap1 and SDHB for sprint performance in humans, likely linked to their role in acute and chronic redox regulation during high-intensity exercise. Keap1 emerges as a potential sensor of the glycolytic rate in skeletal muscle to mediate specific adaptations critical for sprint performance.

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AUTHOR CONTRIBUTIONS

The contributions of the authors are as follows: conception and design of the study: VGA, MMR, and JALC; Collection, analysis, and interpretation of data: ALL co-authors; Drafted the manuscript: VGA, MMR, and JALC; Critically evaluated and contributed to the manuscript: ALL co-authors. All authors have approved the final version of the manuscript.

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Figure legends

Figure 1. Sex differences in basal skeletal muscle protein expression. Antioxidant enzymes (41 M, 10 F) and RONS-sensing proteins (51 M, 10 F) (a), proteins involved in muscle energy metabolism and oxygen transport (51 M, 10 F) (b), and proteins involved in regulation of sarcoplasmic calcium concentration (51 M, 10 F) (c). The extremes of the whiskers represent the limits of the 5th and 95th percentiles, respectively; the thick and thin horizontal lines inside the boxes correspond to the mean and median values, respectively; and the lower and upper limits of the box delimit the 1st and 3rd quartiles, respectively. The statistical analysis was performed with logarithmically transformed data, when appropriate. Males are represented by blue circles and females by pink triangles. * $P<0.05$ compared to males.

Figure 2. Representative immunoblots. Protein expression levels (Western Blot) and a total protein loading control staining (Reactive Brown Staining) of tested proteins from a single female (F) and four male (M) subjects. CON, control samples. Molecular weights for the proteins under examination are indicated on the right side of the blot. The images are organized as follows: SERCA1, SERCA2, phosphofructokinase1 (PFKM), calsequestrin1 (CSQ1), calsequestrin2 (CSQ2), citrate synthase, ATP5A, UQCRC2, SDHB, COXII, NDUFB8, myoglobin, pSer^{16}/Thr^{17} phospholamban and Reactive Brown are presented in duplicate (Panel A). Total Nrf2, pSer^{40} Nrf2, Keap1, Catalase, Glutathione reductase, SOD1, SOD2, and Reactive Brown are displayed in Panel B.

Table 1. Physical characteristics, ergospirometric variables and myosin heavy chain composition (mean±SD).

	Males (n=51)	Females (n=10)	*P*
Age (years)	22.8 ± 2.9	22.1 ± 1.6	0.449
Height (cm)	175.9 ± 7.3	162.0 ± 4.5	0.000
Weight (kg)	74.0 ± 8.6	57.8 ± 7.0	0.000
% body fat	18.2 ± 5.3	27.1 ± 3.5	0.000
Lean body mass (kg)	57.2 ± 5.7	39.6 ± 3.3	0.000
LLM (kg)	19.8 ± 2.1	14.3 ± 1.5	0.000
HRmax (Beats·min^{-1})	192.1 ± 7.7	196.0 ± 6.8	0.133
VO_2max (mL·min^{-1})	3591.2 ± 454.2	2363.0 ± 226.2	0.000
VO_2max (mL·kg^{-1}·min^{-1})	48.9 ± 6.7	41.1 ± 2.9	0.000
VO_2max (mL·kg LLM^{-1}·min^{-1})	182.2 ± 25.2	166.6 ± 15.6	0.018
Wmax (W)	289.9 ± 54.4	193.2 ± 26.8	0.000
MHC I (%)	39.0 ± 13.6	54.4 ± 12.2	0.002
MHC IIa (%)	48.5 ± 10.5	35.7 ± 6.9	0.001
MHC IIx (%)	12.5 ± 9.1	9.9 ± 7.0	0.403

LLM: lean mass of the lower extremities; HRmax: maximal heart rate; VO_2max: maximal oxygen uptake; Wmax: maximal intensity during the incremental exercise test to exhaustion; MHC: myosin heavy chain composition; P-values from t-test for independent groups.

Table 2. Wingate test performance (mean±SD).

	Males (n=51)	Females (n=10)	*P*
Wpeaki (W)	984 ± 173	751 ± 117	0.000
Wmean (W)	562 ± 84	402 ± 61	0.000
Wpeaki (W·kg BW^{-1})	13.3 ± 2.1	13.1 ± 2.2	0.744
Wmean (W·kg BW^{-1})	7.6 ± 0.9	7.0 ± 1.2	0.069
Wpeaki (W·kg LM^{-1})	17.2 ± 2.8	19.0 ± 2.6	0.071
Wmean (W·kg LM^{-1})	9.8 ± 1.2	10.2 ± 1.4	0.430
Wpeaki (W·kg LLM^{-1})	49.6 ± 7.4	52.8 ± 7.1	0.215
Wmean (W·kg LLM^{-1})	28.3 ± 2.9	28.3 ± 4.0	0.980

Wpeaki: instantaneous peak power output; Wpeak1-s: peak power output for 1-s averages; Wmean: mean power output. BW: body weight; LM: whole body lean mass; LLM: lean mass of the lower extremities. P-values from t-test for independent groups.

Table 3. Predictive models for peak power output during sprint exercise (Wingate test).

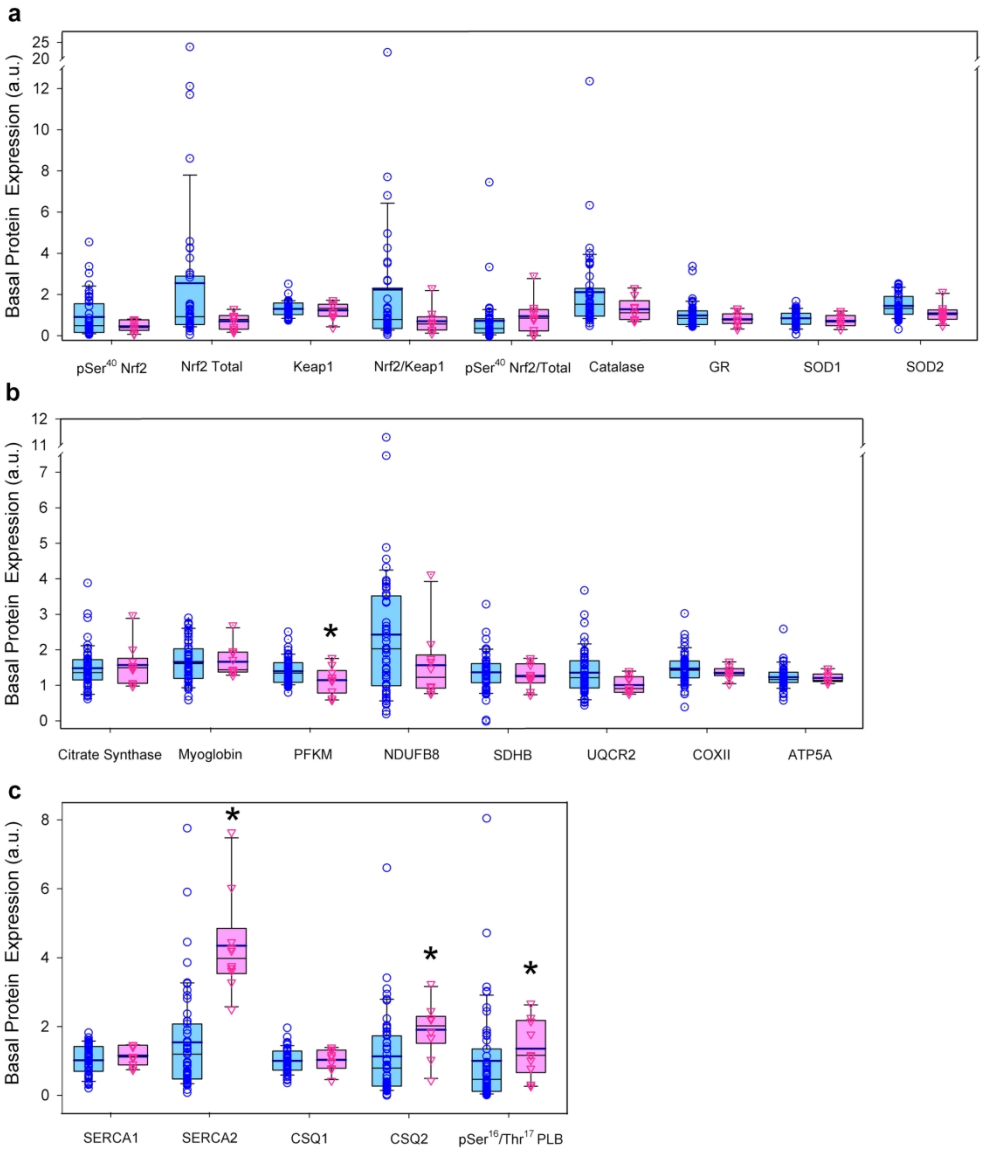
		95% Confidence Interval						95% Confidence Interval			Model fit measures		
Predictor	**Estimate**	**SE**	**Lower**	**Upper**	**t**	**p**	**Stand. Estimate**	**Lower**	**Upper**	**Model**	**R**	**R^2**	***P***
Intercept	41.39	209.5	-380.84	463.62	0.198	0.844							
LLM (kg)	42.7	10	22.55	62.84	4.272	**< .001**	0.647	0.3416	0.9518	1	0.689	0.475	**< .001**
MHC II (%)	4.14	1.41	1.29	6.99	2.93	**0.005**	0.304	0.0948	0.5127	2	0.720	0.518	**< .001**
Keap1 (a.u.)	-137.99	49.15	-237.04	-38.94	-2.808	**0.007**	-0.266	-0.4576	-0.0752	3	0.749	0.562	**< .001**
Log SDHB (a.u.)	196.82	79.55	36.49	357.15	2.474	**0.017**	0.25	0.0464	0.4538	4	0.790	0.624	**< .001**
Sex (1=male; 2=female)	65.07	72.86	-81.76	211.91	0.893	0.377	0.326	-0.41	1.0626	5	0.794	0.631	**< .001**

LLM, lower extremities lean mass; MHC II, myosin heavy chain II percentage; Keap1, kelch-like ECH-associated protein 1; SDHB, succinate dehydrogenase [ubiquinone] iron-sulfur subunit. Power is predicted in watts.

Table 4. Predictive models for mean power output during sprint exercise (Wingate test).

		95% Confidence Interval						95% Confidence Interval			Model fit measures		
Predictor	**Estimate**	**SE**	**Lower**	**Upper**	**t**	**p**	**Stand. Estimate**	**Lower**	**Upper**	**Model**	**R**	**R^2**	***P***
Intercept	-247	142.53	-532.4	38.4	-1.733	0.089							
LLM (kg)	27.2	3.63	19.93	34.5	7.487	**< .001**	0.79	0.57842	1.001	1	0.821	0.675	**< .001**
Log MHC II (%)	150.9	73.44	3.8	297.9	2.054	**0.045**	0.169	0.00425	0.334	2	0.834	0.696	**< .001**
Sex (1=male; 2=female)	12.9	29.16	-45.48	71.3	0.443	0.66	0.129	-0.45463	0.713	3	0.835	0.697	**< .001**

LLM, lower extremities lean mass; MHC II, myosin heavy chain II percentage. Power is predicted in watts.



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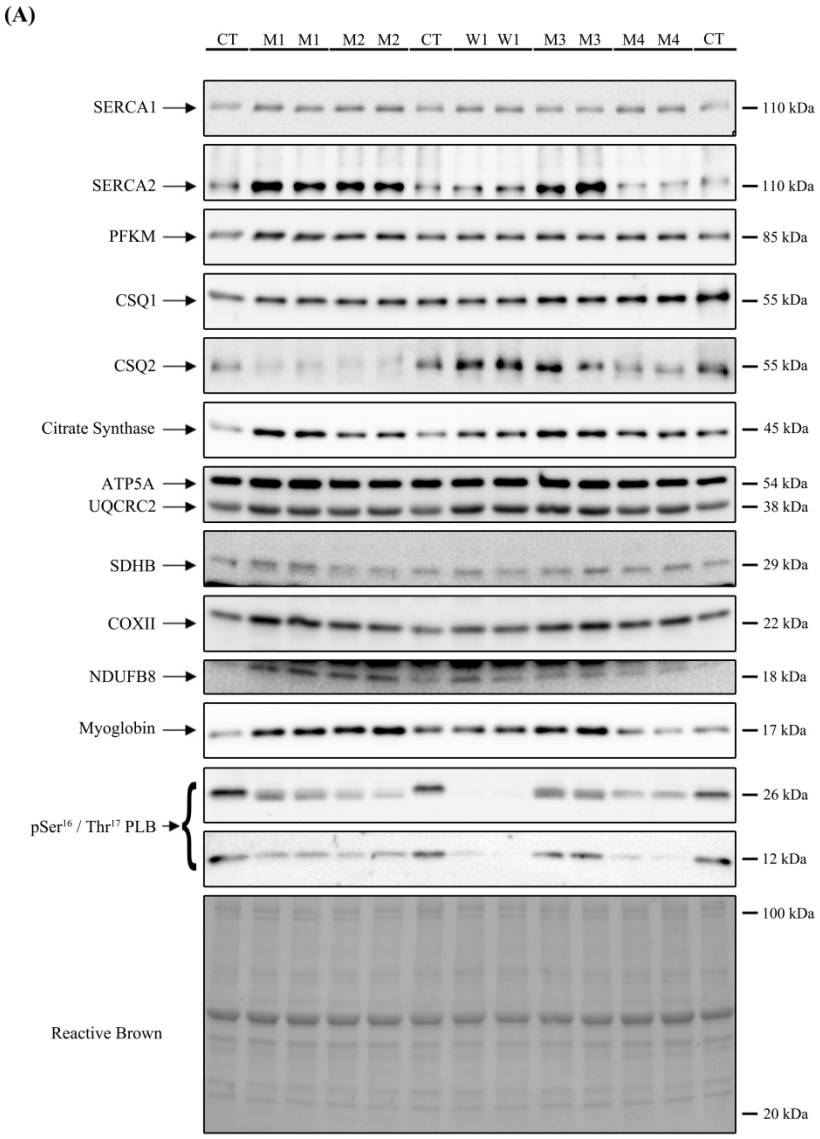

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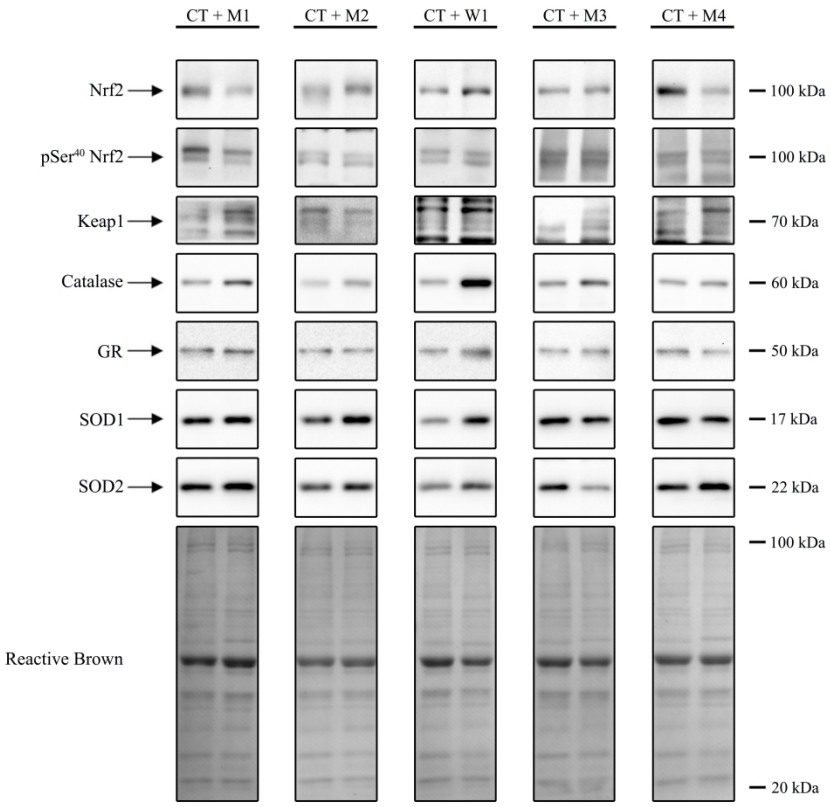

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Supplementary Table 1: Detailed description of Western blotting antibodies and procedures.

Antibody	Manufacturer company	Catalog number	Protein molecular weight (kDa)	Gel %	Protein amount loaded (µg)	Blotting transfer time (min)	Blocking reagent	Primary antibody concentration	Secondary antibody concentration
Nrf2	Abcam	ab62352	100	12.5	12.5	90	BSA 4%	1:1500	1:5000
pSer40 Nrf2	Abcam	ab76026	100	10	15	90	BSA 4%	1:5000	1:5000
Keap1	Proteintech	10503-2-AP	70	10	7.5	90	BSA 4%	1:3000	1:10000
Catalase	Cell Signaling	14097	60	10	12.5	90	Blotto 5%	1:2000	1:5000
Glutathione reductase (GR)	Proteintech	18257-1-AP	50	10	10	90	Blotto 5%	1:2000	1:10000
SOD1	Abcam	ab16831	17	15	12.5	90	BSA 4%	1:2000	1:5000
SOD2	Cell Signaling	13141	22	15	12.5	90	BSA 4%	1:20000	1:10000
Calsequestrin1	Sigma-Aldrich	C0618	55	10	1.5	90	Blotto 5%	1:30000	1:20000
Calsequestrin2	Sigma Aldrich	C3868	55	12.5	3	90	BSA 4%	1:5000	1:5000
SERCA1	Sigma-Aldrich	WH0000487M1	110	10	1.5	90	Blotto 5%	1:50000	1:20000

SERCA2	Sigma-Aldrich	S1439	110	10	3	90	BSA 4%	1:5000	1:5000
Phosphofructokinase1 (PFKM)	Proteintech	55028-1-AP	85	10	1.5	90	BSA 4%	1:3000	1:20000
NDUFB8 (CI)	Abcam	ab110411	18	4-20	8.5	90	Blotto 5%	1:2000	1:5000
SDHB (CII)	Abcam	ab110411	29	4-20	8.5	90	Blotto 5%	1:2000	1:5000
UQCRC2 (CIII)	Abcam	ab110411	38	4-20	8.5	90	Blotto 5%	1:2000	1:5000
COXII (CIV)	Abcam	ab110411	22	4-20	8.5	90	Blotto 5%	1:2000	1:5000
ATP5A (CV)	Abcam	ab110411	54	4-20	8.5	90	Blotto 5%	1:2000	1:5000
Myoglobin	Cell Signaling	D2F5X	17	15	0.3	60	BSA 4%	1:20000	1:50000
Citrate Synthase	Proteintech	16131-1-AP	45	10	1.5	90	BSA 4%	1:3000	1:10000
$pSer^{16}/Thr^{17}$ Phospholamban	Cell Signaling	8496S	12-26	15	3	45	BSA 4%	1:2000	1:5000

ESTUDIO 4

Accurate assessment of walking energy expenditure in the main seafront walking route of Las Palmas de Gran Canaria to promote health-related tourism

Evaluación precisa del gasto energético de la ruta peatonal del paseo marítimo de Las Palmas de Gran Canaria para promover el turismo relacionado con la salud

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Assessment of walking energy expenditure at Las Canteras beach

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Resumen

El objetivo de esta investigación fue determinar el gasto energético al caminar en la ruta peatonal más popular de Las Palmas de Gran Canaria. Se determinó la energía gastada en la ruta urbana principal a pie de Las Palmas de Gran Canaria (13 km en total, ida y vuelta) con un calorímetro indirecto portátil (COSMED K5) en 33 voluntarios de 21-69 años de edad (22 hombres y 11 mujeres). Se utilizó la geolocalización para determinar la velocidad de marcha (5.6 ± 0.5 y 5.2 ± 0.4 km.h^{-1} , para hombres y mujeres, P = 0.044) y el gasto energético exacto correspondiente a cada sector kilométrico y crear paneles informativos de interés para los caminantes para promover Turismo de salud. El gasto energético total (10.8 ± 1.0 y 10.4 ± 0.8 kcal.kg peso corporal^{-1} , respectivamente, P = 0.35) y las respuestas fisiológicas (frequencia cardíaca y variables respiratorias) durante las caminatas fueron similares en ambos sexos. El gasto energético y la contribución de la oxidación de grasas al gasto energético total aumentaron de forma similar con la distancia recorrida en ambos sexos. En conclusión, el gasto energético al caminar aumenta con la distancia recorrida y es similar para hombres y mujeres cuando se normaliza a la masa corporal total. La calorimetría indirecta portátil se puede utilizar para mapear con precisión el gasto de energía asociado con las rutas a pie para promover el turismo de salud.

Palabras clave: Ejercicio, obesidad, turismo de salud, actividad física.

Abstract

This investigation aimed to determine the energy expenditure incurred by males and females in the most popular walking route in Las Palmas de Gran Canaria using state-of-the-art methods. The energy expended in the main walking route of Las Palmas de Gran Canaria (13 km in total, round trip) was determined with a portable indirect calorimeter (COSMED K5) in thirty-three volunteers aged 21-69 yr (22 males and 11 females). Geolocalisation was used to determine the walking speed (5.6 ± 0.5 and 5.2 ± 0.4 km.h^{-1} , for males and females, p=0.044) and exact energy expenditure corresponding to each kilometric sector and create information panels of interest for walkers to promote health tourism. The total energy expenditure (10.8 ± 1.0 and 10.4 ± 0.8 kcal.kg body weight^{-1} , respectively, P = 0.35) and physiological responses (heart rate and respiratory variables) during the walks were similar in males and females. The energy expenditure and the contribution of fat oxidation to the overall energy expenditure increased similarly with the distance walked in both sexes. In conclusion, the energy expenditure for walking routes increased with the distance walked and is similar for males and females when normalized to whole-body mass. Portable indirect calorimetry can be used to accurately map the energy expenditure associated with walking routes to promote health tourism.

Keywords: Exercise, obesity, health-related tourism, physical activity.

Introduction

Vacation periods are associated with significant weight gains persisting over time (Cooper & Tokar, 2016; Yanovski et al., 2000). Therefore, tourists may be interested in burning calories to reduce their body fat or to compensate for some culinary excesses during the holidays could be keen on receiving some information regarding the amount of energy expended during the walks (Michimi & Wimberly, 2012) to adopt better-informed decisions regarding their calorie intake and plan their daily physical activity. However, no attempt has been made to quantify the energy required in specific walking routes, beyond the information provided by phone applications or distance covered, which depends on the terrain characteristics and the velocity of walking, among other factors (Looney et al., 2019a; Looney et al., 2019b; Pandolf et al., 1977). Moreover, general equations developed to estimate the energy cost of walking have mostly used indoor (treadmill) data, which underestimate by 2-18% the actual cost of walking observed outdoors on natural terrain (Fattorini et al., 2012).

Health-oriented tourism aims at improving or maintaining health (Ferrer et al., 2016) while enjoying some of the other benefits associated with touristic activity. For example, walking along coastal and marine destinations is appealing for many tourists (Carvache-Franco et al., 2020). This attractiveness may be even more significant when accompanied by additional values, such as clean and well-maintained blue waterfront (Garrett et al., 2019), areas for children's entertainment, stores and restaurants, and the possibility of combining urban and more natural areas in the walking route (Carvache-Franco et al., 2020; Hall & Ram, 2019; Ram & Hall, 2018). In addition, walking routes can be enhanced by adding touristic points of interest (Gomez-Martin, 2019; Worndl et al., 2017), as sightseeing points, informative panels with historical descriptions, and information regarding the surrounding nature.

Therefore, the primary aim of this research was to determine the total energy expenditure incurred by males and females in the walking route that extends along the urban beach of La Playa de Las Canteras (Las Palmas de Gran Canaria, Spain) and continues through the seafront to the wild neighbour beach of La Playa del Confital, ending in La Isleta natural park. Secondary aims were to determine whether the energy expenditure of walking increases with the duration of the walks and whether there are sex differences in the energy expenditure elicited by prolonged walks. This type of information can be incorporated in information panels to enhance the attractiveness of walking routed to promote health-related touristic activities.

Methods

Study design and participants

First, we defined a walking route extending along all the waterfront of la Playa de Las Canteras in Las Palmas de Gran Canaria, one of the city's main attractions. The beach has a wide walking avenue, with terraces, bars, restaurants, and numerous hotels and touristic apartments. In addition to the tourists residing nearby, the beach is often visited by tourists travelling from their residences in other areas of the Island of Gran Canaria as well as by travellers from the cruisers regularly visiting the city de Gran Canaria. The route has 6.5 km and extends from the Plaza de la Música, near the Auditorium Alfredo Kraus (28°07'46.3 "N 15°26'59.4 "W), along the length of the beach until La Puntilla and continues following the pedestrian street Blas de Lezo and the street Rodrigo de Manrique at the seafront, which leads to la Playa del Confital. The route extends all the length of La Playa del Confital, which is a protected wilderness area and finishes at the fence where a military area starts (28°09'58.6 "N 15°26'22.9 "W) (Fig. 1). Most of the route takes place on a flat pavement, while the last kilometre corresponds to a trail with some irregularities but high walkability (see Fig. 1 for more details regarding the altitude profile). Next, we determined accurately the amount of energy needed to complete a round trip in this route. The energy requirements may be influenced by the physical characteristics of the subjects and speed of walking (Ludlow & Weyand, 2017), and factors related to the features of the terrain (Pandolf et al., 1977). An accurate assessment of energy expenditure during prolonged outdoors activities has been a challenge until the developments of high-precision and accurate portable indirect calorimeters equipped with Global Positioning System (GPS), which allows precise geolocalisation and the assessment of the distance covered and the speed of walk. Therefore, we decided to use the most precise and accurate portable indirect calorimeter available for research purposes and recruited thirty-three volunteers of both sexes with different levels of assumed cardiometabolic fitness and a wide variation in the degree of adiposity and age, with volunteers ranging from lean to overweight and moderately obese, and from young to 69 years of age.

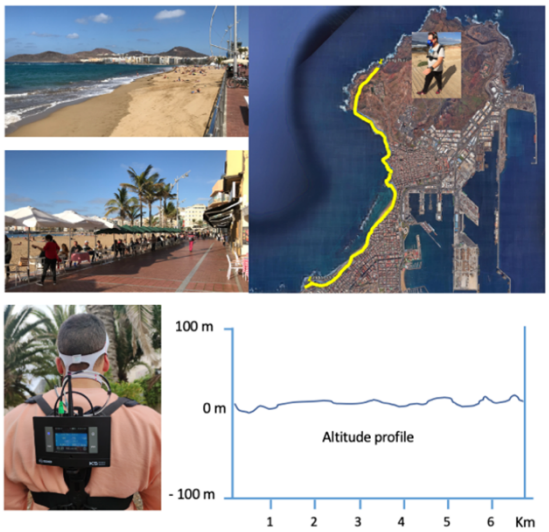

Figure 1. Some pictures of La Playa de Las Canteras walking route and the COSMED K5 device attached on the back of one of the volunteers
The route is marked in yellow, starting at the lowest part of the picture and ending in the upper part.

The study population was composed of fourteen physically active Sports Sciences students (11 male and 3 female) and nineteen sedentary volunteers with overweight or obesity (11 males and 8 females), as defined by a body mass index (BMI) ≥ 25, all of them residents in the Island of Gran Canaria. The general characteristics of males and females are reported in Table 1. Volunteers were accepted in the study under fulfilment of the following inclusion criteria: a) age from 18 to 70 years old; b) BMI > 19 $kg.m^{-2}$; c) no medical contraindications to exercise; d) smoking less than six cigarettes per day, and f) without joint or orthopaedic conditions that could limit their capacity to perform a lengthy walk lasting between 2-3 hours. The study was conducted by the Declaration of Helsinki after ethical approval (Hospital Universitario anonymised ethical committee reference number: 140187). Before giving their written consent, the volunteers received written and oral information regarding the study's purposes, risks, and benefits. In addition, participants were requested to avoid exercise other than their daily usual physical activity and to refrain from caffeinated, carbonated and alcohol- and taurine-containing beverages for the 48 hours preceding the measurements. All volunteers were non-smokers.

Table 1. Characteristics of the study population

	Males (n=22)					Females (n=11)					*p*-value
	Mean	±	SD	Range (min-max)		Mean	±	SD	Range (min-max)		
Age (years) *	33.8	±	12.5	20.9	68.5	39.0	±	10.9	24.9	55.7	.194
Weight (kg)	88.7	±	15.2	64.2	117.9	78.1	±	13.1	58.2	94.3	.060
Height (cm)	176.5	±	6.3	168.4	188.0	164.7	±	5.7	157.2	175.0	< .000
BMI ($kg.m^{-2}$) *	27.2	±	4.5	21.5	35.4	27.7	±	4.9	19.8	33.6	.779
Body fat (%)	26.6	±	8.8	11.8	41.3	38.7	±	8.7	23.1	50.7	< .001
Total lean mass (kg)	58.0	±	6.4	44.3	72.9	42.5	±	4.7	35.7	52.2	< .000
Total fat mass (kg)	23.5	±	11.0	8.7	43.7	29.8	±	10.5	12.9	45.0	.126

Analysis based on an unpaired two-tailed t-test.

***** Statistical analysis performed with logarithmically transformed variables.
BMI: body mass index; SD: standard deviation.

General procedures

Following a 12-h overnight fast, participants reported to the laboratory between 7:00 and 9:30 a.m. to assess their physical characteristics and body composition. First, their body height and mass were measured to the nearest 0.1 cm and 0.1 kg, respectively, while subjects wore light clothes and no shoes, using a balance scale (Seca, Hamburg,

Germany) calibrated using certified calibration masses (class M1, Scheck, Germany). Subsequently, their body fat % and total lean body mass were assessed using a dual-energy X-ray absorptiometer (Lunar iDXA, General Electric, Wisconsin, USA) (Martin-Rincon et al., 2020).

On a different day, the energy expenditure during a round trip (13 km) extending the total distance of the walking route (6.5 km) was assessed between 7:00 and 14:00 after a 12-h overnight fast. For this purpose, the volunteers were equipped with a portable indirect calorimeter (COSMED K5, Rome, Italy) (Fig. 1). The portable indirect calorimeter measures oxygen uptake (VO_2), carbon dioxide production (VCO_2), respiratory exchange ratio, respiratory rate and pulmonary ventilation. This device also records environmental conditions (temperature and barometric pressure) and geolocalisation data (GPS). The environmental conditions were similar during the different walking days. The environmental temperatures ranging from 15 to 29 °C (mean ± SD: 22 ± 2 °C) with a low intra-day variation in temperature (in general less than 2-3 °C) while barometric pressures ranged between 756 to 771 mmHg (mean ± SD: 766 ± 3 mmHg). The accuracy and precision of the COSMED K5 were determined by comparison with a state-of-the-art stationary metabolic cart (Vyntus CPX, Jaeger-CareFusion, Hochberg, Germany) at exercise intensities eliciting a similar energy demand as that measured during walking (Perez-Suarez et al., 2018). The validity and reliability of the stationary Vyntus CPX were previously established using a butane combustion test and repeated measurements (Perez-Suarez et al., 2018). Before the tests, the COSMED K5 was warmed up for a minimum of 15 min and then calibrated with high-grade calibration gases provided by the manufacturers. The flowmeter, used to measure pulmonary ventilation, was calibrated with a 3 L calibration syringe, following the manufacturers' recommendations (COSMED, 2015). After calibration, the K5 was attached to the back of the subjects using a harness, and a face mask of appropriate size was carefully adjusted to avoid gas leaks. The reliability of the COSMED K5 was established in this same route by performing two measurements in fourteen volunteers separated by at least four days. The COSMED K5 was operated in the mixing chamber mode during the walks.

The walks were performed on non-raining days. Volunteers we asked to maintain a walking speed close to 5 $km.h^{-1}$. For this purpose, subjects were equipped with a heart rate strap (Garmin Forerunner 210, Garmin International Inc., Olathe, KS, USA) equipped with GPS connected to the K5. All volunteers were weighed immediately before and after walking (SECA 869, Hamburg, Germany) while wearing all equipment and clothes. The scale was calibrated with certified calibration masses of class M1. Participants were allowed to drink plain water *ad libitum* during the walks.

Calculation of the energy expenditure

Gas exchange data were averaged every 15 seconds and stored for further analysis. From the VO_2 and VCO_2 values, fat and carbohydrate oxidation rates and the energy expenditure were calculated using Peronnet and Massicotte tables (1991). These values were used to calculate the energy expenditure corresponding to each km interval and the entire round trip. Additional calculations were carried out adjusted for the bodyweight of the volunteers.

Statistical analysis

Data are reported as the mean (±SD) unless otherwise stated. Values were checked for normal distribution using the Shapiro-Wilks test. The BMI was determined as weight/ $height^2$. BMI and age were not normally distributed; therefore, these two variables were logarithmically transformed before further analyses. Student's t-tests for unpaired samples were run to determine between-sex differences in physical characteristics and overall energy expenditure. The impact of the distance covered on energy expenditure was determined with analysis of variance for repeated measures with one within-subjects factor (time, with 13 levels corresponding to each kilometre walked) and one between-subjects factor (sex, with two levels). The Mauchly's test of sphericity was run before the ANOVA. In the case of violation of the sphericity assumption, the degrees of freedom were adjusted according to the Huynh and Feldt correction. When a significant main effect or interaction was observed, specific pairwise comparisons were carried out with the Fisher's Least Significant Difference post-Hoc test. The statistical significance was accepted for .-values < 0.05. All statistical analyses were performed using IBM SPSS v.21.0 (IBM, New York, USA).

Results

The descriptive characteristics of the twenty-two males and eleven females that participated in the study are reported in Table 1. In addition, males and females had comparable age and BMI, while females had a greater body fat percentage than males. Males were taller and had a greater whole-body lean mass than females.

As shown in Table 2, the physiological responses were similar in males and females during the walks, as reflected by the heart rate and respiratory response, which were almost identical. Nevertheless, females walked at a marginally lower speed compared to males. Despite the marked differences in physical characteristics, the energy expenditure was similar in males and females (10.8 ± 1.0 and 10.4 ± 0.8 kcal.kg body $weight^{-1}$, respectively, p= .35). The total amount of carbohydrates and fats oxidized during the walks was similar in both sexes, after accounting for the differences in body mass (Table 2).

Table 2. Energy consumption and physiological responses to a round trip in La Playa de Las Canteras-Confital walking route (13 km)

	Men (n=22)					Women (n=11)					*p*-value
	Mean	±	SD	Range (min-max)		Mean	±	SD	Range (min-max)		
Walking velocity (km.h^{-1})	5.6	±	0.5	4.8	6.4	5.2	±	0.4	4.6	5.7	.044
Stride cadence (steps.min^{-1})	60.0	±	3.8	54.0	66.8	60.4	±	2.5	57.3	65.5	.517
Heart rate (beats.min^{-1})	113.0	±	17.3	81.0	143.7	110.0	±	15.1	86.5	133.6	.627
Respiratory rate (breaths.min^{-1})	29.5	±	6.9	20.4	50.1	28.3	±	5.0	22.4	37.0	.610
Total EE (kcal)	952	±	178	722	1400	810	±	119	572	959	.024
Total EE (kcal. kg body mass^{-1})	10.8	±	1.0	9.1	12.9	10.4	±	0.8	9.1	11.6	.345
CHO burned (mg. kg body mass^{-1})	1759	±	450	769	2581	1489	±	226	1218	2005	.072
Fat burned (mg. kg body mass^{-1})	407	±	149	162	727	479	±	67	393	612	.065

EE: Energy expenditure; CHO: carbohydrates. Analysis based on two-tailed unpaired t-tests.

The amount of energy consumed after each km is reported in Table 3 and Fig. 2. Interestingly, the energy cost of walking increased with the distance covered ($p < .001$), being 9.2% higher in the first km sector during the return trip compared to that observed in this same sector at the start of the walk ($p < .001$). Likewise, the percentage of energy obtained from fat oxidation increased from 37.6 ± 14.8 to 55.1 ± 10.8 % from the first km to the same parkour in the return trip ($p < .001$).

Table 3. Energy consumption along the first 6 kilometers of La Playa de Las Canteras-Confital walking route at 5 km.h-1.

	Males body weight				
	60 kg	70 kg	80 kg	90 kg	100 kg
WAPI [a]	Energy expenditure in Kcal				
Km 1	45	52	60	67	75
Km 2	46	54	61	69	77
Km 3	47	55	63	70	78
Km 4	51	60	68	77	85
Km 5	53	61	70	79	88
Km 6	55	64	73	82	91
Total [a]	646	754	861	969	1077
Females body weight					
	50 kg	60 kg	70 kg	80 kg	90 kg
WAPI [a]	Energy expenditure in Kcal				
Km 1	37	37	37	37	37
Km 2	38	38	38	38	38
Km 3	38	38	38	38	38
Km 4	41	41	41	41	41
Km 5	41	41	41	41	41
Km 6	44	44	44	44	44
Total [a]	521	521	521	521	521

Data generated using the mean energy expenditure per kg of body weight recorded during the route for males and females, respectively.

WAPI: Walker's Point of Interest for walkers.
a Total: corresponds to the round trip (13 km).
Precise geolocalisation information available on request from the authors.

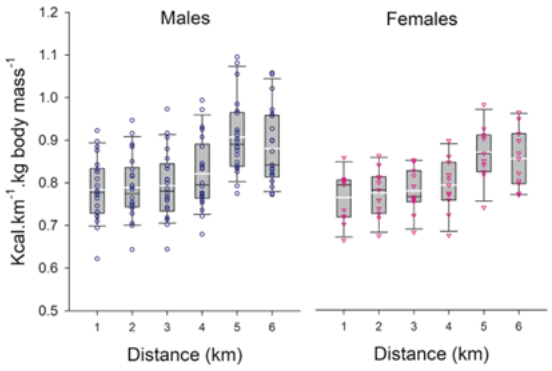

Figure 2. Mean energy expenditure in each km sector, after averaging the outgoing and the return trips for the first six kms.
Box and whisker plots and individual data for males (n = 22) and females (n = 11). The extremes of the whiskers represent the limits of the 5th and 95th percentiles, respectively; the white and black horizontal lines inside the boxes correspond to the mean and median values, respectively; and the lower and upper limits of the box delimit the 1st and 3rd quartiles, respectively. *p* = .345 for the difference between males and females (unpaired two-tailed t-test).

Discussion

This study shows that the energy expended during long outdoors walks is mostly determined by walkers' body weight and the distance covered for walking velocities

ranging between 4.6 and 5.8 $km.h^{-1}$. These walking velocities elicit similar physiological responses and energy expenditure in males and females when adjusted for body weight.

Another aspect of interest is that we have demonstrated that the energy requirements increase slightly with the distance covered. Although this study is the first research to report such observation during prolonged walking, previous studies have seen a similar phenomenon in long-distance runners likely caused by mitochondrial respiration changes (Sahlin et al., 2010). Several published reviews on the mechanisms by which energy expenditure may be increased by prolonged exercise mention oxidative stress, hyperthermia and muscle damage. A detailed analysis of the physiological mechanisms explaining this response is beyond the scope of the present manuscript, and therefore the readers are addressed to several excellent reviews on this topic (Enoka, 2012; Nybo & Nielsen, 2001; Westerblad & Allen, 2011).

Although the energy expenditure of walking can be estimated using prediction equations or the Compendium of Physical Activities (Ainsworth et al., 2011), this may deviate from the actual energy expenditure, which depends in part on the duration of the walk and the characteristics of the terrain, as shown in the present investigation. For example, had we used the Compendium of Physical Activities (Ainsworth et al., 2011) we would have underestimated the actual energy expenditure of the whole route by 2.4 and 13% in males and females, respectively.

Accurate assessment of energy expenditure to promote health-related tourism

The touristic industry is greatly influenced by factors affecting lifestyle and health. Therefore, a continuous adaptation to the needs and wills of consumers is required to offer the best experience to visitors, including the possibility of satisfying their wish for a healthy lifestyle, in which physical activity is a central cornerstone. This entails applying evidence-based knowledge from related disciplines such as economy, sociology, psychology and health sciences to innovate (Darbellay & Stock, 2012). Here we have applied state-of-the-art techniques used in exercise physiology to measure the energy expenditure incurred during prolonged walks in the main walking avenue of one of the most relevant touristic destinations in Europe. We have also provided the stakeholders with information on how to carry out this type of assessment and provided a practical table (Table 3) with material that could be included in what we called "walker's points of interest " (WAPI) in specific information panels, mobile devices, apps, webpages or leaflets for tourists.

In a recent report, The European Travel Commission brings attention to the societal lifestyle trends that shape consumer attitudes, emphasising how "health tourism is expanding its purview to respond to the consumer's hunger for new ways to self-improve". This organisation postulates that health is a prominent issue in tourism, which encompasses travel-friendly concepts such as mental and emotional wellbeing, spiritual growth, adventure, and athleticism, among others (The European Travel Commission, 2016). A particular emphasis is placed on the fact that physical fitness is vital for a large part of the population and that weight and appearance management is a significant-top priority for the three Atlantic markets. Customers want to be healthy, but they also wish to look healthy (The European Travel Commission, 2016). Thus, tourism organisations should market destinations taking into consideration that destinations promoting a physically active lifestyle will be more appealing for most consumers, including the growing elderly population (Diekmann et al., 2020; Ferrer et al., 2016). Moreover, according to The European Travel Commission report, those clients more interested in travelling also confer high importance to their fitness (The European Travel Commission, 2016). Given the recommendations by the medical associations (Arnett et al., 2019; Powell et al., 2018) and the impact of social media messages regarding the importance of physical activity for health, growing demand for wellness offerings within the travel packages are expected in the coming years (Diekmann et al., 2020; Ferrer et al., 2016).

The reasons tourists decide to walk are very varied and encompass social and cultural aspects, a search for adventure, physical and mental health, wellbeing, contact with nature and wilderness, pilgrimage and spirituality, and others (Buckley, 2019, 2020; Carvache-Franco et al., 2020). Distinctive destination marketing can be achieved by creating routes for walkers and cyclists and adding energy expenditure values based on scientific evidence (Davies, 2018). For example, a trail including sightseeing points (Davies, 2018), some adventure (Bichler & Peters), blue spaces (Carvache-Franco et al., 2020; Garrett et al., 2019), nature-based wildlife observation points or areas (Carvache-Franco et al., 2020), architectural and historical attractions, accompanied by maps and short stickers-type messages may enhance the attractiveness and the possibility of creating a one-off experience. Here we have generated the information required to include in these WAPIs data on energy expenditure. These WAPIs could include a map of the route and some recommendations regarding walking velocity, hydration, and the number of calories burned depending on the distance covered (see Fig. 3, as an example). These WAPIs could use pre-existing points or new small panels, which could also have information with links to apps or QR codes, specifically developed to facilitate this information in several languages, if not included in leaflets at the hotels/resorts. In the case of Las Palmas de Gran Canaria, this would add to the great effort made to provide active alternatives to the city bus tours and promote urban routes profiting from pedestrianisation. During holidays, there is more tendency to walk and enjoy the culinary virtues of the destination. Increasing physical activity with some specific aims for energy expenditure would also allow the customers to feel less guilty regarding potential food excesses (Cohen & Avieli, 2004) to achieve a more satisfying holiday experience.

Figure 3. Representation of a Walker's Point of Interest with the information of the energy expenditure accumulated after the 1st, 2nd, 3rd and 4th km.

Conclusions

This investigation has shown that the energy expenditure of walking outdoors increases with the distance covered and is similar for males and females when normalized to whole-body mass, despite remarkable differences in body composition. Portable indirect calorimetry can be used to accurately map the energy expenditure associated with walking routes to promote health tourism. The information gathered can be used to create points of interest for walkers with accurate information regarding the energy expenditure corresponding to each km sector and recommendations for walking speeds.

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