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Impact of exercise and bioactive ingredients on novel cardiometabolic risk markers and energy metabolism

Impacto del ejercicio e ingredientes bioactivos sobre marcadores noveles de riesgo cardiometabólico y el metabolismo energético

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Impact of exercise and bioactive ingredients on novel cardiometabolic risk markers and energy metabolism

Francisco Javier Osuna Prieto

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A mi madre, ejemplo de perseverancia

A mi padre, por regalarme la curiosidad

"Knowing the answers will help you in school.

Knowing how to question will help you in life."

Warren Berger

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LIST OF ABBREVIATIONS

AA: arachidonic acid
ALP: alkaline phosphatase
AMPK: adenosine monophosphate–activated protein kinase (AMPK)
ASHRAE: refrigerating and air conditioning engineers
ATPIII: National Cholesterol Education Program Adult Treatment Panel III
AUC: area under the curve
BA: bile acid
BAT: brown adipose tissue
BMI: body mass index
BMR: basal metabolic rate
CA: cholic acid
cAMP: cyclic adenosin-monophosphate
CDCA: chenodeoxycholic acid
CIT: cold-induced thermogenesis
CMR: cardiometabolic risk
COMT: catechol-O-methyl transferase
CON: control group
CRF: cardiorespiratory fitness
CVD: cardiovascular disease
DCA: deoxycholic acid
DGLA: dihomo-γ-linolenic acid
DHC: dihydrocapsiate
DIT: diet induced thermogenesis
DNA: deoxyribonucleic acid
EE: endurance exercise
EE: energy expenditure
EFSA: European Food Safety Authority
EGCG: epi-gallocatechin gallate
EX-MOD: moderate-exercise intensity group
EX-VIG: vigorous-exercise intensity group
FATox: fat oxidation
FDR: false discovery rate
FFQ: food frequency questionnaire
FLI: fatty liver index
FMI: fat mass index
FNDC5: type I membrane protein
FXR: farnesoid X receptor
GCA: glycocholic acid
GCDCA: glycochenodeoxycholic acid
GDCA: glycodeoxycholic acid
GGT: gamma-glutamyl transferase
GLCA: glycolithocholic acid
GLP1: glucagon-like peptide 1
GLUT4: glucose transporter 4

GPBAR1: G-protein–coupled bile acid receptor 1
GTP: glutamic pyruvic transaminase
GUCDA: glycoursodeoxycholic acid
Hb: haemoglobin
HDL-C: high density lipoprotein cholesterol
HFD: high fat diet
HOMA: homeostatic model assessment [of insulin resistance]
HRR: heart rate reserve
IFN-γ: interferon gamma
IL-6: interleukin 6
LCA: lithocholic acid
LC-MS: liquid chromatography-tandem mass spectrometry
LDL-C: low density lipoprotein cholesterol
LMI: lean mass index
MCT1: monocarboxylate transporter 1
MFO: maximal fat oxidation
MHOO: metabolically healthy overweight/obese
mRNA: messenger ribonucleic acid
MUOO: metabolically unhealthy overweight/obese
NEFAs: non-esterified fatty acids
NIRtrs: near-infrared time-resolved spectroscopy
NST: non-shivering thermogenesis
OGTT: oral glucose tolerance test
PCR: polymerase chain reaction
PET/CT: positron emission tomography combined with computed tomography
PKA: protein kinase A
PUFA: polyunsaturated fatty acids
QC: quality controls
RDP: ribosomal database project
RE: resistance exercise
RM: 1 repetition maximum
RMR: resting metabolic rate
RNA: ribonucleic acid
ROS: reactive oxygen species
RPE: reported perceived exertion
RSD: relative standard deviation
SDH: succinate dehydrogenase
SIRT1: sirtuin 1
SNS: sympathetic nervous system
SPSS: Statistical Package for the Social Sciences
SUV: standardized uptake value
SUVmean: mean standardized uptake value
SUVpeak: peak standardized uptake value
T2D: type 2 diabetes
TC: total cholesterol
TCA: tricarboxylic acid cycle

TG: triglycerides
TGR5: Takeda-G-protein-receptor-5
TNFα: tumor necrosis factor alfa
TPRPA1: transient receptor potential cation channel subfamily A member 1
TRP: transient receptor potential
TRPM8: transient receptor potential melastatin 8
TRPV1: transient receptor potential vanilloid subtype 1
UCP1: uncoupling protein 1
UDCA: ursodeoxycholic acid
VAS: visual analogue scales
VAT: visceral adipose tissue
VCO2: volume of carbon dioxide production
VO2 peak.: peak volume of oxygen consumption
VO2max: maximum oxygen consumption
WAT: white adipose tissue
WC: waist circumference
WHO: World Health Organization
β-AR: beta-adrenergic receptor

ABSTRACT

Obesity and cardiometabolic disease rates are increasing across young and middle-aged adults. This situation calls for the identification and implementation of novel cardiometabolic risk (CMR) markers for identifying individuals at higher risk of developing cardiometabolic diseases and establishing adequate prevention and treatment strategies. In this regard, exercise interventions and the use of bioactive ingredients are both promising strategies to combat obesity and cardiometabolic diseases.

The present International Doctoral Thesis aimed to evaluate the impact of exercise on novel CMR markers (**Section I**) and the impact of bioactive compounds and exercise on energy metabolism (**Section II**). Findings from the studies included in **Section I** revealed that plasma succinate levels might be a promising novel CMR marker in young, sedentary adults (**Study I**). However, succinate levels were not modified after a 24-week of an exercise training program (**Study I**). We also studied the effects of acute endurance and resistance exercise on plasma levels of plasma bile acids (BA), which have been also proposed as novel CMR factors in young adults (**Study II**). This study demonstrated that plasma levels of BA rapidly decreases after a bout of endurance and resistance exercise in an exercise-type specific manner in young, sedentary adults. Remarkably, those individuals with higher cardiorespiratory fitness levels showed a unique response of unconjugated primary BA 120 min after endurance exercise that seems to be reflective of their better health status in comparison to their low cardiorespiratory fitness levels counterparts (**Study II**). Studies from **Section II** concluded that the level of evidence on the use of bioactive ingredients to activate brown adipose tissue (BAT) and to promote white adipose tissue (WAT) browning in healthy humans is weak and scarce (**Study III**). Nevertheless, there is strong scientific evidence from rodent models that supports the use bioactive ingredients to activate BAT and promote WAT browning and thus to potentially combat obesity and cardiometabolic disorders (**Study IV**). Finally, in **Study V** we evaluated the effect of 12 mg of dihydrocapsiate during endurance exercise on energy metabolism, showing that the ingestion of dihydrocapsiate does not increase energy expenditure or fat oxidation during endurance exercise in men with overweight/obesity.

In summary, the present International Doctoral Thesis provides new insights into the impact of acute and long terms effects of exercise on the circulating levels of novel CMR markers. Furthermore, the use of bioactive ingredients is a promising strategy to activate BAT in individuals with obesity and cardiometabolic diseases, while their beneficial effects during exercise remains to be further explored.

RESUMEN

La incidencia de obesidad y enfermedades cardiometabólicas está aumentado en los adultos jóvenes y de mediana edad. Esta situación requiere de la identificación e implementación de nuevos marcadores de riesgo cardiometabólico (RCM) que permitan identificar a aquellos individuos con mayor riesgo de desarrollar enfermedades cardiometabólicas y poder establecer estrategias de prevención y tratamiento de manera temprana. En este sentido, los programas de ejercicio y el uso de ingredientes bioactivos constituyen estrategias prometedoras para combatir la obesidad y las enfermedades cardiometabólicas.

La presente Tesis Doctoral Internacional tuvo como objetivo evaluar el impacto del ejercicio sobre marcadores noveles de RCM (**Sección I**) y el impacto de los ingredientes bioactivos y el ejercicio en el metabolismo energético (**Sección II**). Los hallazgos de los estudios de la **Sección I** revelaron que los niveles de plasmáticos de succinato podrían ser un nuevo y prometedor marcador de RCM en adultos jóvenes sedentarios (**Estudio I**). Sin embargo, los niveles de succinato no se modificaron después de 24 semanas de un programa de entrenamiento (**Estudio I**). En el **Estudio II** estudio demostramos que los niveles plasmáticos de ácidos biliares disminuyen rápidamente después de una sesión de ejercicio de agudo una manera específica y dependiente del tipo de ejercicio (aeróbico o fuerza) en adultos jóvenes sedentarios. Aquellos individuos con mayores niveles de capacidad cardiorrespiratoria mostraron respuesta una característica en los ácidos biliares primarios tras finalizar el ejercicio aeróbico que parece reflejar su mejor estado de salud en comparación con sus los individuos con menores niveles de capacidad cardiorrespiratoria (**Estudio II**). Los estudios de la **Sección II** concluyeron que el nivel de evidencia sobre el uso de ingredientes bioactivos para activar el tejido adiposo pardo (TAP) y promover el "amarronamiento" del tejido adiposo blanco (TAB) en humanos sanos es bajo (**Estudio III**). Sin embargo, existe una importante evidencia científica derivada de estudios en roedores que respalda el uso de ingredientes bioactivos para activar TAB y promover el amarronamiento del TAB para combatir la obesidad y los trastornos cardiometabólicos (**Estudio IV**). Finalmente, en el **Estudio V** evaluamos el efecto de 12 mg de dihidrocapsiato durante una sesión de ejercicio aeróbico, concluyendo que la ingesta de dihidrocapsiato no aumenta el gasto energético ni la oxidación de grasas durante el ejercicio de aeróbico a intensidad FATmax en hombres con sobrepeso/obesidad.

En resumen, la presente Tesis Doctoral Internacional proporciona nuevos conocimientos acerca del impacto de los efectos agudos y crónicos del ejercicio sobre los niveles circulantes de marcadores noveles de RCM. Además, el uso de ingredientes bioactivos es una estrategia prometedora para activar el tejido adiposo pardo en personas con obesidad y enfermedades cardiometabólicas, mientras que sus efectos beneficiosos durante el ejercicio aún deben explorarse más a fondo.

GENERAL INTRODUCTION

OBESITY AND CARDIOMETABOLIC DISEASES: A GLOBAL EPIDEMIC

Obesity has become a global epidemic that significantly reduces life expectancy [1]. It is a well-known fact that obesity is an independent risk factor for cardiometabolic diseases such as insulin resistance, dyslipidemia, hypertension, and sleep disorders [2]. Obesity also leads to the development of cardiovascular disease independently of other cardiometabolic risk (CMR) factors [3]. Nowadays, cardiometabolic diseases are the leading cause of death worldwide [4] and the rates are alarmingly increasing across young and middle-aged adults [5]. Worryingly, individuals with obesity not only experience cardiometabolic complications at an earlier age, but they also suffer cardiometabolic diseases for a larger period of their lifetime and present a reduced life span in comparison with normal-weight individuals [6].

To date, identification of apparently asymptomatic, young individuals at high CMR is yet a major challenge in primary prevention [7]. Therefore, the identification and implementation of novel CMR markers may yield valuable insights into the pathophysiology and prevention of obesity and cardiometabolic diseases, improving the identification of individuals at risk of developing cardiometabolic complications as early as possible to establish adequate prevention and treatment strategies [8–10].

IDENTIFICATION AND IMPLEMENTATION OF NOVEL CARDIOMETABOLIC RISK MARKERS: A PREVAILING NECESSITY

Due to the gradually developing nature of cardiometabolic diseases, a significant fraction of the young population is currently being classified at low CMR according to the current guidelines and criteria [11]. This could be partially explained because these classification systems involve traditional CMR markers such as dyslipidemia, smoking, and hypertension that might be not so effective for identifying young individuals at early stages of cardiometabolic complications [11,12]. For example, dyslipidemia is routinely evaluated through levels of total cholesterol, high-density lipoprotein cholesterol(HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides. Nevertheless, it is known that a significant proportion of individuals with cardiometabolic complications present these lipid/lipoprotein levels within the normal ranges [13]. This suggests that using these traditional CMR markers may not be useful to assess CMR across all population age-groups, particularly across young and relatively healthy adults where these traditional CMR markers are unlikely to be already altered or are within normal clinical values. Moreover, the exclusive use

of this cluster of lipid species (i.e., total cholesterol, HDL-C and LDL-C and/or triglycerides) might not reflect the tremendous complexity of lipid metabolism in humans [14]. For these reasons, advances in the use of "omics" techniques, namely genomics, transcriptomics, proteomics and metabolomics, constitute a promising approach to the identification of endogenous novel circulating CMR markers and to unveil their role in metabolism [15].

Nevertheless, there is evidence that certain molecules that were identified decades ago and that have been profoundly studied, such as the intermediates of the tricarboxylic acid cycle (TCA), could be linked to obesity and cardiometabolic alterations in unforeseen ways. Actually, recent evidence revealed that some of the TCA intermediates could also act as signaling molecules that control the hypoxic response, immunity, DNA modifications, angiogenesis, and even cancer [16]. Among these intermediates, succinate has emerged as a novel pleiotropic signaling metabolite which is produced by both host and succinate-producer bacteria. Several studies have demonstrated that plasma succinate is elevated in several pathological conditions, including individuals with obesity and cardiometabolic alterations [17]. Moreover, surgical weight loss intervention lowered circulating succinate levels in patients with type 2 diabetes and morbid obesity [18,19], being the preoperative circulating levels of succinate an strong predictor of diabetes remission after bariatric surgery [19]. Given this, it has been proposed that circulating succinate levels could be serve as a novel CMR maker in middle-aged adults [20]. However, no study has investigated whether circulating succinate levels could serve as a novel CMR marker in young-adults and its relationship with both traditional and novel (i.e., omega-6 oxylipins) CMR markers.

Metabolomics: disentangling the complexity of human metabolism

After the complete sequencing of the human genome, most of the attention focused to postgenomic technologies, such as transcriptomics, proteomics and metabolomics. The implementation of these technologies have allowed to obtain an integrated view of metabolism by offering new insights into the components that contribute to cardiometabolic diseases [21].

Metabolomics constitutes a field of omics that comprises the characterization of metabolites and metabolic pathways in biological systems (**Fig. 1**). The metabolic phenotyping of an individual allows to use the metabolite readouts as a proxy for an organism's observable biochemical traits and phenotype. Thus, the implementation of metabolomics has already revealed previous unsuspected CMR markers and potential therapeutic strategies [22]. Within metabolomics, lipidomics – that refers to the comprehensive profiling of

lipids species and its metabolic pathways through liquid chromatography - mass spectrometry (LC-MS) techniques – has become one of the most used techniques for studying human metabolism [23]. Lipidomics enabled the quantification of a wide-spectrum of oxidized lipids in just one analytical run, revealing that oxylipins - the oxidized products of omega-3 and omega-6 polyunsaturated fatty acids (PUFA) - might serve as indicators of oxidative stress in chronic diseases and as potential novel CMR markers [24,25]. Interestingly, recent evidence revealed that the plasma levels of a set of four omega- 6 oxylipins may serve as early CMR markers in young adults, since they were positively associated with adiposity levels, prevalence of metabolic syndrome, and elevated circulating glucose and lipid levels [26]. Interestingly, further pathway analyses revealed that those individuals with obesity had higher plasma levels of omega- 6 and lower plasma levels of omega- 3 oxylipins in comparison to their normal-weight counterparts [26].

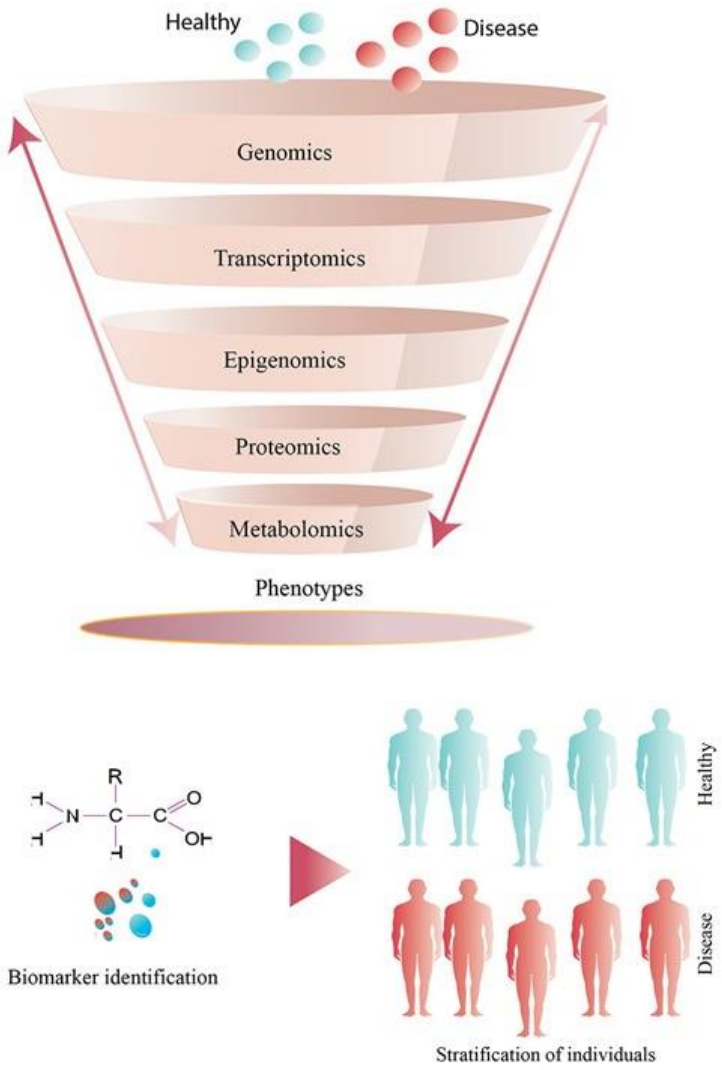

Figure 1: Use of metabolomics for the identification of novel cardiometabolic risk factors and clinical stratification of individuals. Adapted from Sen P et al. Front Mol Biosci 2018.

Lipidomics also cover other molecules that play important roles in human metabolism, such as bile acids. Similar to oxylipins, there is evidence that increased levels of circulating bile acids [27] are linked to cardiometabolic diseases in humans. Actually, recent evidence suggests that circulating bile acids levels could serve as potential novel CMR makers in young individuals, since plasma levels of bile acids were associated with traditional CMR markers in young, relatively healthy adults [28].

Gut microbiota: the importance of the prokaryote universe within us

The gut microbiota is composed by a complex and dynamic population of microorganisms along the gastrointestinal, including bacteria, viruses, fungi and archaea [29]. Among these group of organisms, bacteria are the most abundant and they exert a marked impact on health and disease [30]. Gut microbiota composition is regulated by intrinsic factors such as host genotype and age [31], but it is also affected by extrinsic factors, such as diet and exercise [32,33]. The metabolites produced by these bacteria modulate biological host processes such as digestion and absorption of nutrients, immunity and gut barrier permeability [34,35].

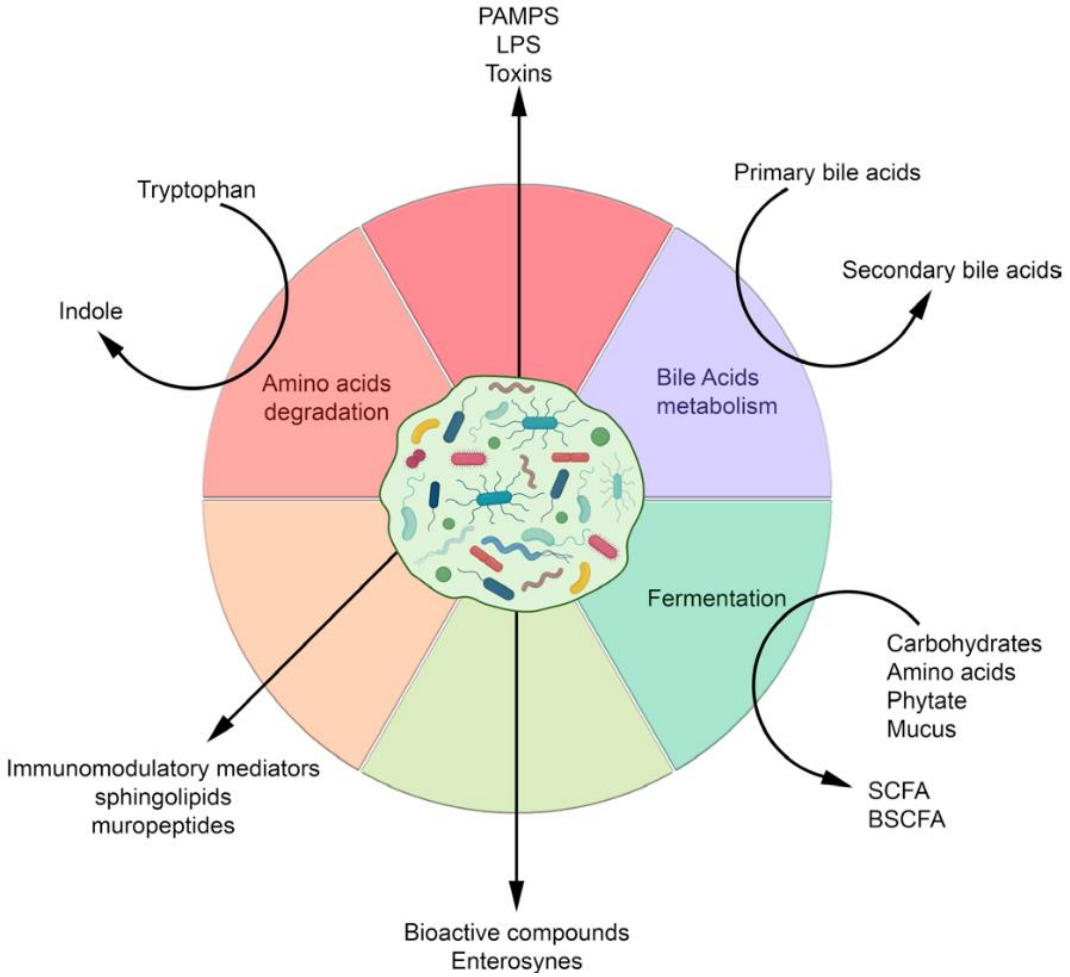

Figure 2. Example of molecules and metabolites produced by the gut microbiota in relation to the nutrients or metabolic source and their derived compounds. BSCFA, branched SCFA; LPS, lipopolysaccharides; PAMPs, pathogen- associated molecular patterns; SCFA, short chain fatty acids. de Vos WM et al. Gut 2022.

Alterations in the composition of gut microbiota have been linked to the development of obesity and cardiometabolic diseases in humans [36,37]. Indeed, certain bacteria metabolites such as trimethylamine-n-oxide [36], secondary bile acids [36], or even some TCA intermediates, such as succinate [17], are linked to obesity and cardiometabolic diseases (**Fig. 2**). In this context, the modification of extrinsic factors that modulates gut microbiota composition in a beneficial fashion, such as exercise and diet, are promising strategies to restore the functionality of gut microbiota [38–40]. Consequently, including the assessment of gut microbiota composition in clinical studies could help to get a better understanding of the mechanisms that lead to obesity and cardiometabolic diseases.

STRATEGIES TO COMBAT OBESITY AND CARDIOMETABOLIC DISEASES: A FOCUS ON EXERCISE AND BIOACTIVE INGREDIENTS

Unequivocally, the implementation of novel CMR markers will improve the screening of individuals at high risk of developing cardiometabolic diseases [41]. Besides, it is important to consider that CMR markers not only serve to evaluate the CMR status of the individuals, but also to evaluate and monitoring the impact of interventions aiming to combat obesity and cardiometabolic diseases [42,43]. To date, most of non-pharmacological interventions for combating obesity and cardiometabolic diseases advocate for recommending guidelines for physical activity healthy dietary habits, like the World Health Organization guidelines on physical activity and sedentary behaviour [44].

Exercise interventions to counteract obesity and promote cardiometabolic health

Growing epidemiological evidence indicates that sedentary behaviour is associated with all-cause and cardiometabolic morbidity and mortality [45]. Indeed, physical inactive individuals present an increased prevalence of cardiometabolic diseases, cancer, and early mortality [46], whereas physically active individuals present an better glucose (i.e., lower glucose levels, higher insulin sensitivity) and lipid (i.e., lower LDL-C, lower triglycerides, higher HDL-C) profile, and lower blood pressure levels in comparison to physical inactive individuals (**Fig. 3**) [47]. In this sense, exercise is an effective strategy to improve cardiometabolic health in both healthy and unhealthy individuals [48]. Several meta-analyses have demonstrated that endurance exercise training decreases blood pressure levels in hypertensive individuals [49,50], improves lipid

metabolism in patients with hyperlipidaemia [51,52], reduces glycated haemoglobin in patients with type 2 diabetes [51], improves body composition in obese individuals [51], and reduces intrahepatic fat [53].

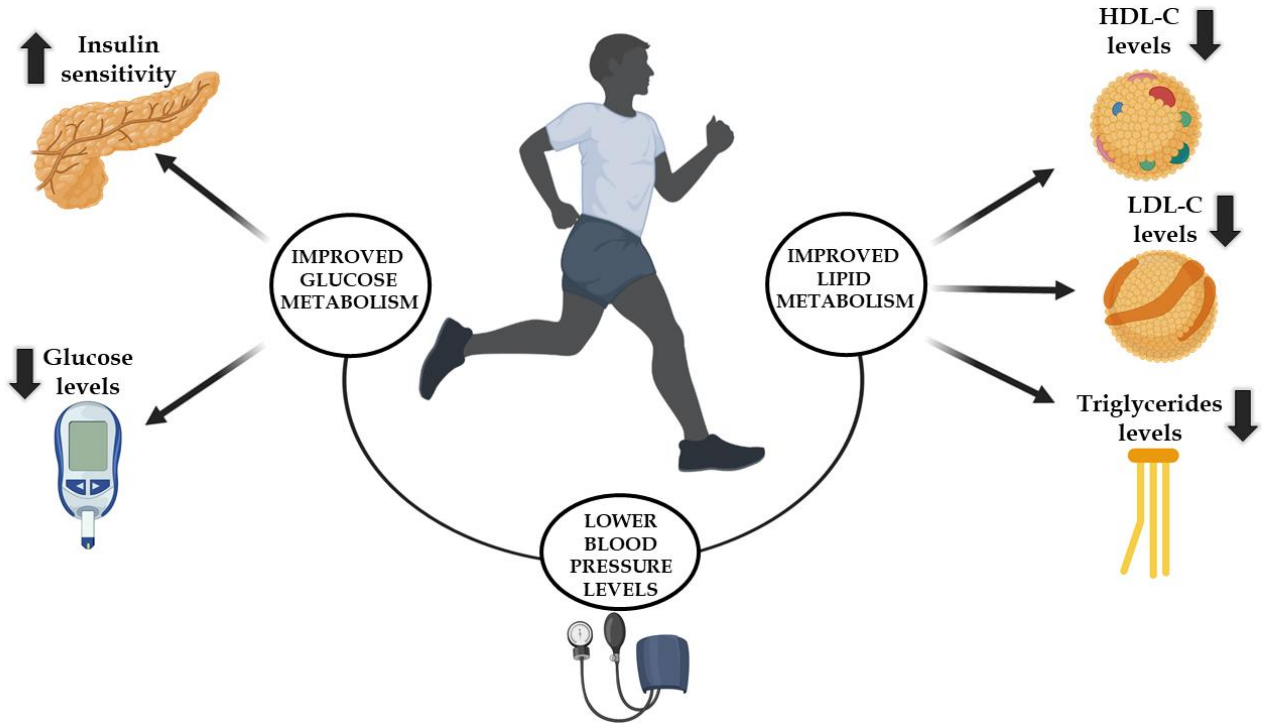

Figure 3. Beneficial effects of exercise on cardiometabolic health in humans.

Nonetheless, despite the effectiveness of exercise training on improving cardiometabolic health in adults with overweight/obesity [53,54], little is known on the effects of exercise training on traditional and novel CMR in populations of young and apparently healthy individuals. Thereby, further research is warranted to understand the effects of exercise on novel CMR markers, such as circulating TCA intermediates or bile acids in young adults.

Metabolic lessons from a single bout of exercise: clinical interest of studying the acute effects of exercise

Beyond the role of exercise training in promoting cardiometabolic health, the study of the acute effects of exercise provides unique metabolic insights into the identification of early stages of metabolic disease and molecules that could serve to evaluate the response to therapeutic interventions [55,56].

Traditionally, most of the studies that aimed to investigate the changes in circulating molecules in response to exercise focused on the measurement of a relatively small number of molecules, usually as a result of the intrinsic

limitations of the techniques required. Nowadays, the implementation of multi-omics approaches to study the effects of acute exercise have shown that a bout of exercise modifies the concentration of a myriad circulating molecules that are involved in the regulation of several physiological process, such as energy metabolism, inflammation and oxidative stress [57]. However, it is important to consider that endurance and resistance exercise have a different impact on human physiology [58]. Endurance exercise leads to cardiovascular adaptations that increase peak oxygen consumption with none/little effects on strength parameters, whereas resistance exercise improves neuromuscular functions that lead to significant improvements in strength and muscle mass with none/little impact on peak oxygen consumption [59]. These specific exercise-type responses are mediated by a complex interplay between a several signaling pathways coupled to metabolic downstream effectors [60]. Actually, the metabolomic profiling comparing the responses of acute endurance exercise and acute resistance exercise revealed that they do impact different metabolic pathways, a phenomena that is linked to an specific plasma metabolite fingerprint [61].

Therefore, a better understanding of the impact of different types of acute exercise (i.e., endurance and resistance) on novel CMR markers could lead to improvements in the identification of early stages of cardiometabolic diseases that might be unnoticed through routing assessments of CMR markers (i.e., fasting levels), and to evaluate the response to therapeutic interventions, such as exercise and diet.

Bioactive ingredients to combat obesity and cardiometabolic diseases

A definition of bioactive ingredients was adopted by consensus in the 23rd Hohenheim Consensus Meeting, defining bioactive ingredients as essential and non-essential compounds that naturally occur in small amounts in food and plants and have shown to exert positive effect(s) on human health [62]. Throughout history, humans have relied on natural products containing bioactive ingredients to counteract disease. In fact, the structure and properties of some of these bioactive ingredients have led to the development of a wide list of drugs that are currently being used in the prevention and treatment of obesity and cardiometabolic diseases [63,64]. Evidence from epidemiological studies support that high intake of bioactive ingredients through fruits and vegetables is associated with a reduced incidence of obesity and cardiometabolic diseases. Bioactive ingredients combat obesity and cardiometabolic diseases by targeting different pathways and regulatory function that can lead to increases in energy

expenditure and satiation, inhibition of the pancreatic lipase activities, or reduction of circulating glucose and/or lipid levels [65].

Interestingly, it seems that some bioactive ingredients increase energy expenditure through the activation of non-shivering thermogenesis (NST), which is defined as the increase of metabolic heat production above the basal metabolism without the involvement of muscle shivering.

Bioactive ingredients targeting non-shivering thermogenesis

Brown adipose tissue (BAT) is a thermogenic organ that generates heat via non-shivering thermogenesis (NST) to maintain body temperature. BAT thermogenesis is supported by the action of the uncoupling protein 1 (UCP1) in the mitochondrial inner membrane, the molecular distinctive of BAT (**Fig. 4**) [66]. UCP1 could be also expressed by beige adipocytes, brown-like adipocytes that emerge within white adipose (WAT) depots [67]. The main BAT activator is cold exposure [68]. The cold stimulus activate the transient potential receptor (TRP) channels in the skin, which act as temperature receptors [69]. This results in the activation of the sympathetic nervous system (SNS) and the thermogenic program in brown and beige adipocytes [70]. Both circulating and intra-cellular fatty are used to fuel thermogenesis within BAT mitochondria [71]. Moreover, circulating glucose is also a fuel for of brown adipocytes, allowing imaging techniques to use labelled glucose to trace human BAT activity [72]. Thus, because BAT consumes energy to generates heat, strategies aimed to targeting BAT thermogenesis and/or WAT "browning" (emerging of beige adipocytes within WAT) have arisen as potential therapeutic tools against obesity and cardiometabolic diseases [73].

During the last decade, several studies revealed negative associations of human BAT with body mass index (BMI) [74], body fat mass [75–77], circulating glucose [76,78], total cholesterol and triglycerides [79,80], and with the incidence of type 2 diabetes [81]. In fact, recent evidence has reinforced that BAT presence could be associated with a better cardiometabolic profile in humas [82]. This study included more than 50,000 patients and revealed that individuals with detectable BAT presented lower prevalence of cardiometabolic diseases, i.e., lower odds of type 2 diabetes, dyslipidemia, hypertension and cardiovascular events [82]. Furthermore, individuals with detectable BAT also presented an improved profile of traditional CMR markers, such as lower glucose and triglycerides levels, and higher HDL-C values in comparison with their non-detectable BAT counterparts.

Acute and chronic cold exposure increases BAT volume and activity in humans, and improves metabolic health in obese, and diabetic patients [83–86], yet cold interventions are hard to implement in clinical practice [70,87]. A potential alterative to cold interventions is the use of bioactive ingredients. TRP channels are not only regulated by temperature, but also for bioactive ingredients naturally present in food and plants [88]. Among TRP channels, TRP vanilloid 1 (TRPV1), TRP ankyrin 1 (TRPA1), and TRP Melastin 8 (TRPM8) are the most relevant for BAT activation, as their stimulation is associated with increased BAT activity [89]. For these reasons, bioactive ingredients might be able to mimic the effects of cold exposure and activate BAT through the activation of TRP channels [70]. In addition, the activation of TRPV1, TRPA1, and TRPM8 has demonstrated to prevent obesity and cardiometabolic diseases by inhibiting body fat gain and pro-inflammatory pathways [90–92].

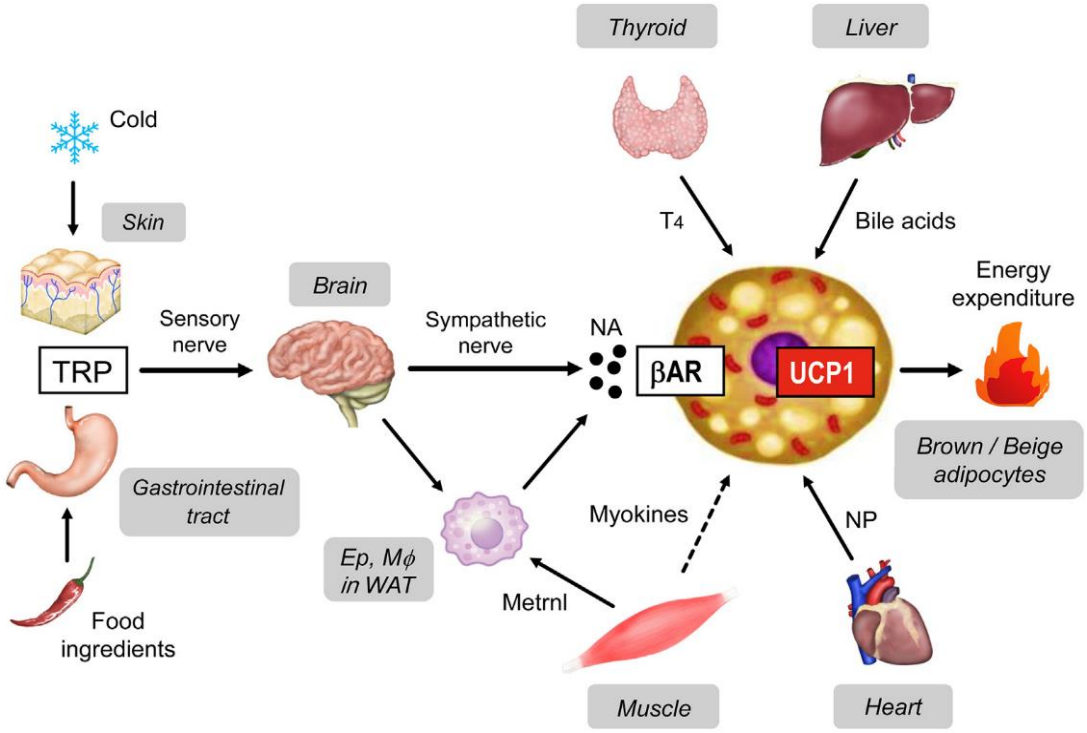

Figure 4. Sympathetic and endocrine control of brown adipose tissue thermogenesis. βAR; beta-adrenergic receptor, Ep; eosinophils, Metrnl; meteorin-like, Mf; macrophage, NA: noradrenaline, NP: natriuretic peptide, TRP; transient receptor potential channel, UCP1; uncoupling protein 1, WAT; white adipose tissue. Saito M et al. Best Pract Res Clin Endocrinol Metab. 2016

Thereby, the activation of BAT and promotion of WAT browning could yield extra benefits beyond the increase in energy expenditure, constituting a promising tool to treat obesity and cardiometabolic diseases in humans [93]. However, further studies should address which bioactive ingredients are the most effective for increasing BAT activity and/or WAT browning in humans in

order to provide an evidence-based list of bioactive ingredients to be used in clinical trials.

Exercise and bioactive ingredients

Despite it has been clearly demonstrated the adherence to exercise and nutrition guidelines is an effective strategy to combat obesity and its cardiometabolic co-morbidities, the adherence to these beneficial life style patters is often low [94]. This fact calls for multi-dimensional approaches that hopefully will lead to a higher adherences and ultimately higher success rates among the population. [95].

Some bioactive ingredients with anti-obesity properties, such as capsaicin, green tea catechins, nitrates, or curcumin, also exert effects that could enhance exercise benefits in humans [96]. Particularly, capsaicin and capsinoids seems to exert their ergogenic effects by activating TRPV1, the same channels responsible for its thermogenic effects [97]. Some of the ergogenic effects mediated by TPRV1 activation are: i) increased calcium release by the sarcoplasmic reticulum of contracting skeletal muscle cells; ii) higher fatty acid oxidation; and iii) promotion of glycogen sparing, which results in an increased aerobic exercise performance [98]. Nonetheless, most of the research on the field has been conducted in trained individuals and/or athletes [96], a population which physiological characteristics and response to exercise significantly differ from individuals with overweight/obesity problems.

While the benefits of endurance exercise have shown to be effective in weight loss and cardiometabolic risk management in adults with overweight/obesity [54], little is known whether these effects could be further enhanced (i.e., higher energy expenditure and at fat oxidation during exercise per bout of exercise) by the combined use of bioactive ingredients and aerobic exercise in adults with overweight/obesity.

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AIMS

GENERAL AIM

The general aim of the present International Doctoral Thesis is to understand the impact of exercise and bioactive ingredients on novel cardiometabolic risk markers and energy metabolism in adults.

SPECIFC AIMS

SECTION I. Impact of exercise on novel markers of cardiometabolic risk markers

- Specific aim I: to investigate the relationship between plasma succinate levels with traditional and novel cardiometabolic risk markers, and to evaluate the effect of a 24-week exercise training intervention on plasma succinate levels in young adults (Study I).

- Specific aim II: to investigate the effects of an acute maximal endurance and resistance exercise on plasma levels of bile acids in young adults (Study II).

SECTION II: Impact of bioactive compounds and exercise on energy metabolism

- Specific aim I: to investigate the effect of bioactive ingredients on brown adipose tissue volume and activity in humans (Study III).

- Specific aim II: to study the effect of bioactive ingredients on brown adipose tissue and white adipose tissue browning in rodent (mice and rats) (Study IV).

- Specific aim III: to evaluate the effects of dihydrocapsiate ingestion on energy expenditure and fat oxidation during an acute bout of aerobic exercise in men with overweight/obesity (Study V).

METHODS

Table 1. Methodological overview of the studies included in the Doctoral Thesis.

Study	Design	Cohort	Participant's characteristics*	Independent variable	Dependent variables	Statistical analyses
Study I	Cross-sectional	The ACTIBATE study	n=100 (♀=65%) Age: 22.0 1 ± 2.6 years BMI: 24.8.0 ± 4.4kg/m^2	- Plasma succinate levels	- Cardiometabolic risk factors - Fecal microbiota composition	- One-way analysis of variance (ANOVA) - Unpaired *t*-tests
Study II	Acute exercise trial	The ACTIBATE study	n= (♀=72%) Age: 21.2 ± 2.5 years BMI: 25.3.0 ± 4.3kg/m^2	- Acute endurance exercise -Acute resistance exercise	- Plasma bile acids levels	- General linear models (repeated measures) - Unpaired *t*-tests
Study III	Systematic review	N.A	N.A	Bioactive ingredients	-BAT activity/volume -RMR -CIT	- Following PRISMA statement[1]
Study IV	Systematic review	N.A	N.A	Bioactive ingredients	- UCP1 expression* in BAT - UCP1 expression* in WAT	- Following PRISMA statement[1]
Study V	Randomized, triple-blinded, placebo-controlled, crossover trial	The ACTIFOX study	n=24 men Age: 40.2 ± 9.2 years BMI: 31.6.0 ± 4.5kg/m^2	- Dihydrocapsiate - Placebo	- Energy expenditure - Fat oxidation	- General linear models (repeated measures) - Paired *t*-tests

*Presented as mean ± standard deviation; ♀: Percentage of women, BAT: brown adipose tissue, BMI: body mass index, CIT: cold-induced thermogenesis, N.A, non-available, RMR: resting metabolic rate; UCP1: uncoupling protein 1, WAT: white adipose tissue

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RESULTS AND DISCUSSION

SECTION I

STUDY I: Elevated plasma succinate levels are linked to higher cardiovascular disease risk factors in young adults

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Cardiovascular Diabetology. 2021 Jul 27;20(1):151. PMID: 34315463

ABSTRACT

Background: Succinate is produced by both host and microbiota, with a key role in the interplay of immunity and metabolism and an emerging role as a biomarker for inflammatory and metabolic disorders in middle-aged adults. The relationship between plasma succinate levels and cardiovascular disease (CVD) risk in young adults is unknown.

Aim: To determine the relationship between plasma succinate levels and traditional and novel CVD risk in young adults.

Methods: Cross-sectional study in 100 (65% women) individuals aged 18–25 years from the ACTIvating Brown Adipose Tissue through Exercise (ACTIBATE) study cohort. CVD risk factors, body composition, dietary intake, basal metabolic rate, and cardiorespiratory fitness were assessed by routine methods. Plasma succinate was measured with an enzyme-based assay. Brown adipose tissue (BAT) was evaluated by positron emission tomography and circulating oxylipins were assessed by targeted metabolomics. Fecal microbiota composition was analyzed in a sub-sample.

Results: Individuals with higher succinate levels had higher levels of visceral adipose tissue (VAT) mass (+ 42.5%), triglycerides (+ 63.9%), C-reactive protein (+ 124.2%), diastolic blood pressure (+ 5.5%), and pro-inflammatory omega-6 oxylipins than individuals with lower succinate levels. Succinate levels were also higher in metabolically unhealthy individuals than in healthy overweight/obese peers. Succinate levels were not associated with BAT volume or activity or with fecal microbiota composition and diversity.

Conclusions: Plasma succinate levels are linked to a specific pro-inflammatory omega-6 signature pattern and higher VAT levels and seem to reflect the cardiovascular status of young adults.

BACKGROUND

Cardiovascular disease (CVD) remains the main cause of death worldwide [1]. Worryingly, the rates of CVD are increasing in young/middle-aged adults (18–45 years) [2]. The incorporation of new circulating biochemical markers and technologies are improving the detection of CVD risk in the general population [3,4]. Early identification of individuals at risk of developing CVD is important [5], but predictive biomarkers of CVD risk and related metabolic disturbances are not well characterized in young adults [6]. Advances in the functional analysis of the human metabolome have yielded many new endogenous metabolites as potential biomarkers for CVD [7], including the tricarboxylic acid (TCA) cycle intermediate succinate [8].

Historically considered as a respiratory substrate of the mitochondrial electron transport chain, succinate is now known to have additional physiological roles. For example, it acts as a signaling molecule in both intracellular and extracellular compartments by binding and activating its cognate receptor, succinate receptor 1 (SUCNR1), also known as G-protein coupled receptor 91 [9]. In addition to being a marker of hypoxia and a driver of tissue damage [10], succinate is now recognized as a pro-inflammatory signal that boosts immune activation [11–13]. We and others have shown that succinate also plays a key role in the fine-tuning of the inflammatory response, acting both as an alarmin [11–13] and as a resolving molecule [14–17]. Moreover, succinate is a positive regulator of intestinal gluconeogenesis [18], activates brown adipose tissue (BAT) thermogenesis [19], and is involved in the muscle-remodeling program in response to exercise [20,21]. Additional roles for succinate in energy metabolism are anticipated from the finding that acute dietary intake modulates post-prandial succinate plasma levels by a mechanism that is dependent on intestinal glucose sensing and metabolic status [22]. Succinate is also a microbiota-derived metabolite with a key role in governing intestinal homeostasis [23]. Succinate levels are clearly elevated in inflammatory-related health conditions, including obesity and type 2 diabetes (T2D) [22,24–26], and are also related to a microbiota dysbiosis signature [26]. Indeed, succinate has been validated as a surrogate biomarker of poor metabolic control in patients with obesity and T2D [22,24,26] and can predict diabetes remission in patients undergoing bariatric surgery [24]. To date, however, no study has investigated whether circulating succinate levels are associated with CVD risk, whether it can be a biomarker of CVD risk [3–6]. Similarly, whether an exercise-intervention program is an effective strategy to decrease circulating succinate levels in young adults remains to be investigated.

Oxylipins are a large family of lipid-based metabolites derived from polyunsaturated fatty acids that differentially regulate inflammatory processes, representing a novel group of putative CVD risk biomarkers [27,28]. Omega-3 oxylipins mainly exert anti-inflammatory and pro-resolving effects, whereas omega-6 oxylipins are mainly involved in pro-inflammatory processes [29,30]. Interestingly, previous work has established a link between higher levels of circulating omega-6 oxylipins and an elevated pro-inflammatory status and CVD risk [31,32], but little is known about the role of omega-3 oxylipins for CVD risk.

In the present study, we aimed to determine the relationship between plasma succinate levels and CVD risk in young adults. We examined whether succinate levels correlate with traditional and novel CVD risk factors (*i.e.*, oxylipins) in a well-phenotyped cohort of young adults. As a secondary aim, we examined the effect of a 24-week supervised exercise-intervention program on plasma succinate levels in young adults.

METHODS

Participants

The present study was conducted within the framework of the ACTIBATE (ACTIvating Brown Adipose Tissue through Exercise) study [33], a randomized controlled trial designed to determine the effect of exercise on BAT activity (Clinical trials identifier: NCT02365129). Inclusion criteria were the following: to be sedentary (<20 min moderate-to-vigorous physical activity on <3 days/week), non-smoker, not taking any medication, and stable body weight over the last 3 months. Exclusion criteria were: diagnosis of diabetes, hypertension or any medical condition(s) that can interfere with or be aggravated by exercise, being pregnant, using medication (including antibiotics) that could affect energy metabolism or gut microbiota, and being frequently exposed to cold temperatures (*e.g.*, indoors/outdoors workspace with low-temperatures, such as cold-storage works, ski/snow monitors, fieldwork during the winter sessions or low-temperature areas). All participants gave their informed consent, and the study was approved by the Ethics Committee on Human Research of the University of Granada (nº.924), and Servicio Andaluz de Salud (Centro de Granada, CEI-Granada). We selected participants from the ACTIBATE study with valid data for serum CVD risk factors, body composition, dietary intake, basal metabolic rate (BMR), cardiorespiratory fitness, brown adipose tissue (BAT) volume and activity, and with plasma samples available for succinate measurements. This resulted in a cohort of 100 young adults (65 women, 35 men; age 18–25 years) that were used in subsequent analyses. Of this cohort, 58

individuals had available fecal samples that were used in the fecal microbiota and composition analysis. **Table 1** shows the descriptive characteristics of the participants as well as the plasma succinate levels measured at baseline. A 24-week randomized exercise-controlled trial was conducted with a parallel-group design. After the baseline examinations, individuals were randomly assigned into three different groups using a computer-generated simple randomization (29): (i) control group (CON, no exercise, n=36), (ii) moderate-exercise intensity group (Ex-MOD, n=32), and (iii) vigorous-exercise intensity group (Ex-VIG, n=32). The study was conducted in two consecutive years in 4 different waves (from September 2015 to June 2016, and from September 2016 to June 2017). All participants were instructed not to change their normal routine and their physical activity and dietary patterns throughout the study.

Procedures

All data were collected at the same hour of the day, but on different days within a period of three weeks. Participants commuted to the research center by car, bus, or motorcycle, and all reported to have slept as usual and refrained from stimulant beverages and any moderate physical activity in the previous 24 h, or any vigorous physical activity in the 48 h prior to each visit. Participants remained still (either lying down or sitting) during the assessments. Self-reported menstrual cycle phase of female participants was recorded at each visit.

Anthropometry, basal metabolic rate, and dual-energy X-ray absorptiometry

On the first visit, participants arrived at 08:15 AM (after a 12-h overnight fast, with a standardized dinner the evening before). Waist circumference was measured twice at the minimum perimeter area with a measuring tape (mm precision) and the mean value was calculated. For those with abdominal obesity, waist circumference was measured just above the umbilicus (horizontal plane). Body mass and height were measured (no shoes, light clothing) using a model 799 Seca scale and stadiometer (Seca, Hamburg, Germany). After having urinated, participants put on standardized clothes (clothing insulation value: 0.20) and entered a warm room (22.8 ± 0.9°C; 43.8 ± 6.7% humidity). Basal metabolic rate (BMR) was measured during 30 min while lying down on a bed using a CCM Express or Ultima CardiO2 metabolic cart (Medical Graphics Cardiorespiratory Diagnostics St Paul, MN) [34,35], according to methodological recommendations [36]. We selected the average of the most stable 5-min period, as it was the most accurate estimation of the individuals' BMR [34]. Body fat mass, lean body mass and visceral adipose tissue (VAT) were measured by whole-body

dual-energy X-ray absorptiometry (HOLOGIC, Discovery Wi, Marlborough, MA). Body mass, lean mass, and fat mass indices were calculated as kg/m^2.

Positron emission tomography-computed tomography scanning and analysis

On the second visit, participants arrived in a fasted condition (≥6 h) and were placed in a cool room (19.5–20°C) wearing a water-perfused cooling vest (Polar Products Inc., Stow, OH) and the same standardized clothes as on visit 1. Water temperature was progressively reduced until shivering occurred (self-reported and visually observable). The water temperature at the onset of shivering was recorded as the shivering threshold (5.4 ± 2.2°C for men and 6.3 ± 2.2°C for women; common range for both sexes is 3.9–12.2°C). At 48–72 h after the shivering threshold test, on visit 3, the participants were placed in a cool room (19.5–20°C) with the cooling vest temperature set at 4°C above their individual shivering threshold. After 1 h of cold exposure with a cooling vest 4°C above their individual shivering threshold and the room temperature at 19.5–20°C, they received an intravenous injection of ~185 MBq ^{18}F-^{18}F-fluorodeoxyglucose (FDG) while the water temperature was increased by 1°C. The positron emission tomography combined with computed tomography (PET-CT) scan was performed one hour after the injection, and scans were analyzed using the Beth Israel plug-in for FIJI software [37], in agreement with the methodological recommendations [38] and following a protocol described elsewhere [39,40]. PET-CT images from cervical vertebra 1 to thoracic vertebra 6 (approximately) were obtained. To assess BAT volume and ^{18}F-FDG uptake, we selected voxels with a radiodensity between −190 and -10 Hounsfield Units and an ^{18}F-FDG uptake above the individualized standardized uptake value (SUV) threshold of 1.2/(lean body mass/body mass) [38]. Based on this information, BAT volume and ^{18}F-FDG uptake (calculated as SUV mean) parameters were obtained following the BARCIST 1.0 recommendations [38].

Cardiorespiratory fitness

On the fourth visit, individuals arrived in fasting conditions (3–5 h) having refrained from drinking coffee/tea during the testing day or the day before. Neither vigorous exercise (48 h before) nor moderate exercise (24 h before) was allowed prior to the assessments. A treadmill maximum-exercise test employing an H/P/Cosmos Pulsar treadmill (H/P/Cosmos Sports & Medical GmbH, Nussdorf-Traunstein, Germany) was performed according to a modified Balke protocol [33]: 1-min warm-up at 3 km/h, followed by 2 min at 4 km/h, and 1 min at 5.3 km/h. Subsequently, the treadmill slope was increased by 1% each minute

until volitional exhaustion was reached. Respiratory gas exchange was monitored with a CPX Ultima CardioO$_2$ system (Medical Graphics Corp., St Paul, MN) with a facemask, model 7400 (Hans Rudolph Inc., Kansas City, MO), and a preVent™ metabolic flow sensor (Medical Graphics Corp.) [34]. Carbon dioxide production (VCO_2) was assessed using a non-dispersive infra-red sensor, and oxygen consumption (VO_2) was measured using a galvanic fuel cell [34]. Maximum VO_2 (VO_2max) was defined as a respiratory exchange ratio of ≥1.1, once a VO_2 plateau was reached, with a heart rate within 10 beats/min of the individuals' age-predicted maximum (209-0.73×age) [41]. VO_2 max was calculated relative to body mass [42].

Cardiovascular disease risk factors and plasma succinate analysis

During the fifth visit, blood samples were drawn from the antecubital vein in the morning (8.00–9.00 A.M) after overnight fasting (>10 h), under resting conditions. Blood samples were collected in Vacutainer Tubes®, which were immediately centrifuged, and serum (obtained with Vacutainer® SST™ II Advance tubes) and plasma (obtained with Vacutainer® Hemogard™ tubes, containing potassium salt of ethylenediamine tetra-acetic as anticoagulant) aliquots were stored at -80°C until analyses. Serum samples were used for cardiovascular risk factor analyses, whereas plasma samples were used to determine succinate and omega-3 and omega-6 oxylipin concentrations.

Glucose was measured in an AU5832 biochemical analyzer (Beckman Coulter, Brea, CA) using a Beckman Coulter reagent (#OSR6521) and insulin was measured in a DXI analyzer (Beckman Coulter) using a Beckman Coulter chemiluminescent reagent (#33410). These values were used to calculate the homeostatic model assessment (HOMA) index of insulin resistance [43]. Total cholesterol, triglyceride, and high-density lipoprotein-cholesterol (HDL-C) serum levels were measured in the AU5832 analyzer using the Beckman Coulter reagents #OSR6116, OSR60118 and OSR6187, respectively. Low-density lipoprotein-cholesterol (LDL-C) levels were subsequently calculated using the Friedewald formula: (total cholesterol) − (HDL-C) − 0.45 * (triglycerides). [44] C-reactive protein was also measured in an AU5832 analyzer with the reagent #OSR6299. Plasma succinate levels were measured using the EnzyChrom™ Succinate Assay Kit (BioAssay Systems, Hayward, CA). The assay sensitivity was 12 μM and the intra- and inter-assay coefficients of variance were <3.50% and 6.95%, respectively, and the accuracy ranged from 1 to 11.5% error [24,26].

Systolic and diastolic blood pressure was measured with an automatic sphygmomanometer (Omrom M2; Omron Healthcare, Kyoto, Japan). Measurements were repeated on three different days and the averages were calculated. The prevalence of metabolic syndrome was calculated according to the National Cholesterol Education Program Adult Treatment Panel III (ATP III) criteria [45] .Participants were considered to have metabolic syndrome if they had three or more of the following risk factors: waist circumference ≥102 cm for men and ≥88 cm for women; triglycerides ≥150 mg/dL; HDL-C <40 mg/dL for men and <50 mg/dL for women; systolic blood pressure ≥130 mmHg or diastolic blood pressure ≥85 mmHg; glucose >110 mg/dL.

Fecal microbiota analysis

On the sixth visit, a fecal sample (50–60 g) was collected from a sub-cohort of n=58 participants using a sterilized plastic container. Samples were transported in a portable cooler at 4°C to the laboratory and stored at -80°C until DNA extraction. Fecal samples were homogenized in a Stomacher® 400 (A. J. Seward and Co. Ltd., London, UK) and DNA extraction and purification were performed with a commercial kit (QIAamp DNA Stool Mini Kit, QIAGEN, Barcelona, Spain). DNA was quantified using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, DE) and quality was evaluated according to the A260/280nm and A260/230nm absorbance ratios.

Purified DNA was amplified by PCR targeting the V3 and V4 hypervariable regions of the bacterial 16S rRNA gene by using the following primer pairs, 16S Amplicon Forward Primer: 50TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCW GCG, and 16S Amplicon Reverse Primer: 50GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTA TCTAATCC. [46] PCR assays were conducted in a final volume of 25 µL, consisting of 12.5 µL 2× KAPA HiFi Hotstart ready mix (KAPA Biosystems, Woburn, MA), 5 µL forward primer (1 µM), 5 µL reverse primer (1 µM), 2.5 µL DNA (10 ng), with the following PCR program: 1) denaturation (95ºC, 3 min); 2) 8 cycles of denaturation (95ºC, 30 s); annealing (55ºC, 30 s) and elongation (72ºC, 30 s); 3) final extension (72ºC, 5 min). Next, AMPure XP beads (Beckman Coulter, Indianapolis, IN) were used to purify the 16S V3 and V4 amplicons. A PCR indexing step was then performed, which attaches dual indices and Illumina sequencing adapters using the Nextera XT Index Kit (Illumina, San Diego, CA). The PCR conditions were as follows: 1) 95ºC, 3 min; 2) 8 cycles of 95ºC, 30 s; 3) 55ºC, 30 s; 4) 72ºC, 30 s; 5) 72ºC, 5 min; 6) hold at 4ºC. Pooled PCR products were

purified using AMPure XP beads (Beckman Coulter) before quantification. Finally, the amplicons were sequenced at MiSeq (Illumina) using paired-end (2×300 nt) Illumina MiSeq sequencing system (Illumina).

Merging and pre-clustering of raw sequences was conducted using the "DADA2" [47] package in R [48], allowing differences in 2 nucleotides (so-called phylotypes), which were filtered according to a threshold for mean abundance of 0.001% and a sequence length 240 pb before the analysis. A total of 11,659,014 paired-end reads were obtained with an average of 126,728±33,395 reads per sample. All samples were above the 10,000 reads cut-off. Samples were standardized to an equal size of 30,982 reads using the "PHYLOSEQ" [49] package in R [48], obtaining a total of 11,158 phylotypes. The "CLASSIFIER" function from the Ribosomal Database Project (RDP) was used for assigning taxonomic affiliation of phylotypes, according to the naive Bayesian classification [50] by using a pseudo-bootstrap threshold of 80%. A total 209 genera belonging to 16 different phyla were obtained. To further determinate the annotation of phylotypes (species assignments), the "SEQMATCH" function from RDP [51] was employed to define the discriminatory power of each sequence read; annotation was conducted according to previously published criteria [52]. Microbial communities were analyzed from phylum to species, calculating relative abundances expressed as percentages for use in subsequent analyses. Only the data for abundances higher than 1% relative abundance were represented at phylum and genus level according to the study variables.

Beta and alpha diversity metrics, and fecal microbiota composition, were then determined and used in the subsequent analyses. Beta diversity indicates differences in microbial community composition between individuals [53], whereas alpha diversity indicates the number of different phylotypes and relative abundances within a given individual [54]. Alpha diversity was calculated based on the Chao richness, inverse Simpson, Camargo's evenness, and Shannon indices with the "MICROBIOME" [55] package in R software [48]. Chao richness estimates the diversity according to the number of different phylotypes identified in the community [56]; Shannon diversity increases as both the richness and the evenness of the community increase [57]; the inverse of Simpson diversity is calculated from classical Simpson diversity and indicates richness in a community with uniform evenness [58]; and Camargo's evenness indicates the equitability of phylotypes frequencies in the community [59].

Data are presented as means ± standard deviations unless otherwise stated. Normality of all variables was assessed using the D'Agostino & Pearson

omnibus with GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, CA). Since variables were non-normally distributed, non-parametric tests were used for all analyses. The cohort was divided into tertiles according to circulating succinate levels (low, intermediate or high groups) using the Statistical Package for the Social Sciences v.22.0 (IBM SPSS Statistics, IBM Corporation, Chicago, IL). The "VEGAN" [60] R package was used for calculating the data matrix comprising the relative abundances at phylum and genus levels using the Bray-Curtis algorithm [61] for measuring beta diversity. Samples were ordinated by principal coordinate analysis. Significance level threshold was set at $P<0.05$. R software (V.3.6.0) [48] and GraphPad Prism were also used for plots. Beta diversity was measured quantitatively for relative abundance higher than 0.5% by permutational multivariate analysis of variance (PERMANOVA) based on Bray-Curtis [61] dissimilarity, with Past3 [62]. The Kruskal-Wallis test was used for the assessment of significant differences in gut microbiota composition and alpha diversity. P-values were corrected by the two-stage step-up method of Benjamini, Krieger and Yekutieli multiple comparison by controlling the False Discovery Rate (FDR).

Dietary recalls

Regular dietary energy intake was estimated using three non-consecutive 24-h dietary recalls, one of which was on a non-working day. Participants were interviewed by dietitians who recorded all food items and drinks that the individuals consumed on the day prior to the interview. The methodology has been extensively described elsewhere [63]. In brief, a book with pictures of different food servings and sizes was used to help participants estimate the amount of food consumed. EvalFINUT® software (http://www.finut.org/evalfinut/) was used to obtain the nutritional composition of the diet, which was used to obtain dietary energy and macronutrients intake, and dietary energy density parameters. Consumption of water and salt was not recorded. Participants were not informed in advance when their diet was going to be registered.

Determination of plasma omega-3 and omega-6 oxylipins

Plasma levels of omega-3 and omega-6 oxylipins were measured and analyzed using a targeted metabolomics approach with liquid chromatography-tandem mass spectrometry (LC-MS/MS), as described elsewhere [64]. Using this LC-MS/MS method, 83 oxylipins were detected and relatively quantified (**Table S1**).

Oxylipins were extracted using liquid-liquid extraction [64]. Briefly, 150 µL of plasma was transferred into a 1.5 mL-Eppendorf tubes and was spiked with 5 µL of a solution of butylated hydroxytoluene (0.4 mg/mL) and 10 µL of a deuterated internal standard mix. Next, 150 µL of a buffer solution (0.2 M citric acid and 0.1 M disodium hydrogen phosphate) were added, followed by the addition of 1000 µL of the extraction solvent methyl tert-butyl ether and butanol (50:50, *v/v*). Samples were mixed for 5 min with a bullet blender (Next Advance, Averill Park, NY), and then centrifugated (16,000 *g*, 10 min, 4°C). After the centrifugation step, 900 µL of the upper layer was transferred to a new 1.5 mL Eppendorf tube. Samples were evaporated to dryness using a SpeedVac system prior to reconstitution in 50 µL of a solution of methanol:acetonitrile (70:30, *v/v*). The resulting solution was centrifuged (16,000 g, 10 min, 4°C), prior to the collection of 40 µL of the supernatant, which was transferred into glass vials for injection in the LC-MS/MS system.

The extracted samples were analyzed using a Shimadzu LC system (Shimadzu Corporation, Kyoto, Japan), coupled to a SCIEX QTRAP 6500+ mass spectrometer (SCIEX, Framingham, MA). Separation was performed using a BEH C18 column (50 mm × 2.1 mm, 1.7 µm) from Waters Technologies (Milford, MA) kept at 40°C. The mobile phase consisted of 0.1% acetic acid in water (A), 0.1% acetic acid in acetonitrile/methanol (90:10, *v/v*, B), and 0.1% acetic acid in isopropanol (C). Ionization was performed using electrospray ionization in negative mode. For the MS/MS acquisition, selected reaction mode (SRM) was employed. SRM transitions were individually optimized for targeted analytes and respective internal standards using standard solutions. The list of internal standards is shown in **Table 1.**

For each target compound detected, the ratio between its peak area and the peak area of its corresponding internal standard was calculated using SCIEX OS Software. Quality control (QC) samples (i.e., blank plasma samples) were used to evaluate the quality of the data and to correct for between-batch variations, using the in-house developed mzQuality workflow (available at http://www.mzQuality.nl). [65] Relative standard deviations (RSDs) of the peak area ratios were calculated for each target analyte present in the QC samples. Metabolites showing RSDs higher than 30% on peak area ratios in QC samples were excluded from further analysis (**Table 1**).

Table 1. List of metabolites analyzed by LC-MS/MS

Abbreviation	IUPAC Name	ChEBI ID	RSD in QC samples
Omega-3 oxylipins			
ALA	9Z,12Z,15Z-octadecatrienoic acid	27432	9.80%
9-HpOTrE	9S-hydroperoxy-10E,12Z,15Z-octadecatrienoic acid	165791	NM
9-HOTrE	9S-hydroxy-10E,12Z,15Z-octadecatrienoic acid	80447	7.60%
12,13-DiHODE	(±)-12,13-dihydroxy-9Z,15Z-octadecadienoic acid	88461	5.80%
EPA	5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid	28364	8.50%
5-HpEPE	5S-hydroperoxy-6E,8Z,11Z,14Z,17Z-eicosapentaenoic acid	145815	NM
5-HEPE	(±)-5-hydroxy-6E,8Z,11Z,14Z,17Z-eicosapentaenoic acid	72801	13.10%
12-HpEPE	12S-hydroperoxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid	78909	NM
12-HEPE	(±)-12-hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid	72645	12.10%
14,15-EpETE	(±)-14,15-epoxy-5Z,8Z,11Z,17Z-eicosatetraenoic acid	88457	NM
14,15-DiHETE	(±)-14,15-dihydroxy-5Z,8Z,11Z,17Z-eicosatetraenoic acid	88459	8.00%
17,18-EpETE	(±)-17,18-epoxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid	72853	NM
17,18-DiHETE	(±)-17,18-dihydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid	88349	9.10%
DPA	7Z,10Z,13Z,16Z,19Z-docosapentaenoic acid	61204	14.00%
DHA	4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid	28125	9.80%
4-HDoHE	(±)-4-hydroxy-5E,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid	72624	14.30%
8-HDoHE	(±)-8-hydroxy-4Z,6E,10Z,13Z,16Z,19Z-docosahexaenoic acid	72610	19.70%
11-HDoHE	(±)-11-hydroxy-4Z,7Z,9E,13Z,16Z,19Z-docosahexaenoic acid	72794	17.10%
13-HDoHE	(±)-13-hydroxy-4Z,7Z,10Z,14E,16Z,19Z-docosahexaenoic acid	72608	12.30%
14-HDoHE	(±)-14-hydroxy-4Z,7Z,10Z,12E,16Z,19Z-docosahexaenoic acid	72647	14.70%
16-HDoHE	(±)-16-hydroxy-4Z,7Z,10Z,13Z,17E,19Z-docosahexaenoic acid	72613	15.40%
17-HDoHE	(±)-17-hydroxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid	72637	9.00%
20-HDoHE	(±)-20-hydroxy-4Z,7Z,10Z,13Z,16Z,18E-docosahexaenoic acid	72615	23.60%
19,20-EpDPE	(±)-19(20)-epoxy-4Z,7Z,10Z,13Z,16Z-docosapentaenoic acid	72653	13.50%
19,20-DiHDPA	(±)-19,20-dihydroxy-4Z,7Z,10Z,13Z,16Z-docosapentaenoic acid	72657	7.90%
Omega-6 oxylipins			
LA	9Z,12Z-octadecadienoic acid	17351	10.20%
10-NO2-LA	10-nitro,9Z,12Z-octadecadienoic acid	34125	13.30%
9-HPODE	(±)9-hydroperoxy-10E,12Z-octadecadienoic acid	165782	NM
9-HODE	(±)-9-hydroxy-10E,12Z-octadecadienoic acid	72651	7.60%
9,12,13-TriHOME	9S,12S,13S-trihydroxy-10E-octadecenoic acid	34506	6.90%
9,10,13-TriHOME	9S,10S,13S-trihydroxy-11E-octadecenoic acid	34499	15.70%
13-HPODE	(±)13-hydroperoxy-9Z,11E-octadecadienoic acid	91272	NM
13-HODE	(±)-13-hydroxy-9Z,11E-octadecadienoic acid	72639	7.10%
9,10-EpOME	9,10-epoxy-12Z-octadecenoic acid	34494	7.80%
12,13-EpOME	(±)-12(13)-epoxy-9Z-octadecenoic acid	38229	9.60%

9,10-DiHOME	9,10-dihydroxy-12Z-octadecenoic acid	72663	7.30%
12,13-DiHOME	12,13-dihydroxy-9Z-octadecenoic acid	72665	6.70%
DGLA	8Z,11Z,14Z-eicosatrienoic acid	53486	23.90%
8-HETrE	8S-hydroxy-9E,11Z,14Z-eicosatrienoic acid	140473	22.80%
15-HETrE	15S-hydroxy-8Z,11Z,13E-eicosatrienoic acid	88348	13.50%
AA	5Z,8Z,11Z,14Z-eicosatetraenoic acid	15843	13.40%
AdrA	7Z,10Z,13Z,16Z-docosatetraenoic acid	53487	22.10%
PGG2	9S,11R-Epidioxy-15S-hydroperoxy-5Z,13E-prostadienoic acid	27647	NM
PGH2	9S,11R-Epidioxy-15S-hydroxy-5Z,13E-prostadienoic acid	15554	NM
PGE2	9-oxo-11R,15S-dihydroxy-5Z,13E-prostadienoic acid	15551	36.00%
PGF2alpha	9α,11α,15S-trihydroxy-prosta-5Z,13E-dien-1-oic acid	15553	ND
TxA2	9S,11S-Epoxy,15S-hydroxy-thromboxa-5Z,13E-dien-1-Oic acid	15627	NM
TxB2	9S,11,15S-trihydroxy-thromboxa-5Z,13E-dien-1-oic acid	28728	6.90%
8,9-EpETrE	8,9-epoxy-5Z,11Z,14Z-eicosatrienoic acid	34490	ND
11,12-EpETrE	11,12-epoxy-5Z,8Z,14Z-eicosatrienoic acid	34130	ND
11,12-DiHETrE	11,12-dihydroxy-5Z,8Z,14Z-eicosatrienoic acid	63969	8.40%
14,15-EpETrE	14,15-epoxy-5Z,8Z,11Z-eicosatrienoic acid	34157	19.50%
5,6-EpETrE	(±)5,6-epoxy-8Z,11Z,14Z-eicosatrienoic acid	34450	NM
5,6-DiHETrE	5,6-dihydroxy-8Z,11Z,14Z-eicosatrienoic acid	63974	9.00%
8,9-DiHETrE	8,9-dihydroxy-5Z,11Z,14Z-eicosatrienoic acid	63970	9.90%
11,12-DiHETrE	11,12-dihydroxy-5Z,8Z,14Z-eicosatrienoic acid	63969	8.40%
14,15-DiHETrE	14,15-dihydroxy-5Z,8Z,11Z-eicosatrienoic acid	63966	7.10%
5-HPETE	5S-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid	91268	NM
5-HETE	5-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid	28209	10.40%
11-HPETE	11R-Hydroperoxy-5Z,8Z,12E,14Z-eicosatetraenoic acid	165279	NM
11-HETE	11-hydroxy-5Z,8Z,12E,14Z-eicosatetraenoic acid	72606	10.70%
12-HPETE	12S-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid	15626	NM
12-HETE	12-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid	19138	11.40%
15-HPETE	15S-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid	91271	NM
15-HETE	15-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid	64017	9.30%
20-HETE	20-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid	34306	9.40%
1a,1b-dihomo-PGF2α	1a,1b-dihomo-9S,11R,15S-trihydroxy-5Z,13E-prostadienoic acid	NA	22.10%
2,3-dinor-8-iso-PGF2α	9α,11α,15S-trihydroxy-2,3-dinor-(8β)-prosta-5Z,13E-dien-1-oic acid	NA	ND
2,3-dinor-11β-PGF2α	9α,11β,15S-trihydroxy-2,3-dinor-prosta-5Z,13E-dien-1-oic acid	NA	ND
iPF2α-IV	(8S)-10-[(1R,2S,3S,5R)-3,5-Dihydroxy-2-pentylcyclopentyl]-8-hydroxydeca-5,9-dienoic acid	NA	ND
5-iPF2α VI	(8β)-5,9α,11α-trihydroxy-prosta-6E,14Z-dien-1-oic acid	140933	ND
8,12-iPF2α	(12α)-5,9α,11α-trihydroxy-prosta-6E,14Z-dien-1-oic acid	NA	7.20%
12-HHTrE	12S-hydroxy-5Z,8E,10E-heptadecatrienoic acid	63977	8.10%
20-hydroxy-PGF2a	9α,11α,15S,20-tetrahydroxy-prosta-5Z,13E-dien-1-oic acid	165322	ND
20-hydroxy-PGE2	9-oxo-11α,15S,20-trihydroxy-prosta-5Z,13E-dien-1-oic acid	137370	ND
8-iso-PGF2a	9α,11α,15S-trihydroxy-(8β)-prosta-5Z,13E-dien-1-oic acid	34509	ND
8-iso-15-R-PGF2a	9α,11α,15R-trihydroxy-(8β)-prosta-5Z,13E-dien-1-oic acid	NA	ND
11beta-PGF2a	9α,11β,15S-trihydroxy-prosta-5Z,13E-dien-1-oic acid	27595	ND
PGF2alpha	9S,11R,15S-trihydroxy-5Z,13E-prostadienoic acid	15553	ND

PGE3	9-oxo-11α,15S-dihydroxy-prosta-5Z,13E,17Z-trien-1-oic acid	28031	ND
PGD3	9α,15S-dihydroxy-11-oxo-prosta-5Z,13E,17Z-trien-1-oic acid	34939	ND
8-iso-PGE2	9-oxo-11α,15S-dihydroxy-(8β)-prosta-5Z,13E-dien-1-oic acid	131888	ND
11beta-PGE2	9-oxo-11β,15S-dihydroxy-prosta-5Z,13E-dien-1-oic acid	89581	ND
PGD2	9α,15S-dihydroxy-11-oxo-prosta-5Z,13E-dien-1-oic acid	15555	ND
8-iso-13,14-dihydro-15-keto-PGF2a	9α,11α-dihydroxy-15-oxo-(8β)-prost-5Z-en-1-oic acid	NA	ND
13,14-dihydro-15-keto-PGF2a	9α,11α-dihydroxy-15-oxo-prost-5Z-en-1-oic acid	63976	ND
13,14-dihydro-PGF2a	9α,11α,15S-trihydroxy-prost-5Z-en-1-oic acid	88346	ND
13,14-dihydro-15-keto-PGE2	9,15-dioxo-11α-hydroxy-prost-5Z-en-1-oic acid	15550	ND
13,14-dihydro-15-keto-PGD2	9α-hydroxy-11,15-dioxo-prost-5Z-en-1-oic acid	72603	ND
1a,1b-dihomo-PGF2a	9α,11α,15S-trihydroxy-1a,1b-dihomo-prosta-5Z,13E-dien-1-oic acid	NA	ND
bicyclo-PGE2	11-deoxy-13,14-dihydro-15-keto-11β,16. xi.-cycloprostaglandin E2	89568	ND
5S,6R-LipoxinA4	5S,6R,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid	6498	ND
5S,6S-LipoxinA4	5S,6S,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid	63990	ND
20-carboxy-LTB4	5S,12R-dihydroxy-6Z,8E,10E,14Z-eicosatetraene-1,20-dioic acid	27562	ND
20-hydroxy-LTB4	5S,12R,20-trihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid	15646	ND
10S,17S-DiHDoHE	10(S),17(S)-dihydroxy-4Z,7Z,11E,13Z,15E,19Z-docosahexaenoic acid	138653	ND
18-HEPE	(±)-18-hydroxy-5Z,8Z,11Z,14Z,16E-eicosapentaenoic acid	72802	ND
15-HEPE	(±)-15-hydroxy-5Z,8Z,11Z,13E,17Z-eicosapentaenoic acid	72627	ND
9-HEPE	(±)-9-hydroxy-5Z,7E,11Z,14Z,17Z-eicosapentaenoic acid	89570	ND

ChEBI: Chemical Entities of Biological Interest; IUPAC, International Union of Pure and Applied Chemistry; NA: not available, ND: not detected, NM: not measured, QC: quality control, RSD: relative standard error.

Classification of individuals into metabolic healthy overweight-obese and metabolic unhealthy overweight-obese groups

Individuals were categorized as metabolic healthy overweight/obese (MHOO; n=27) or metabolic unhealthy overweight/obese (MUOO; n=16) as described [66]. The MHOO group included individuals with a body mass index (BMI) ≥25 kg/m^2 and without any of the following cardiovascular risk factors: i) serum HDL-C <40 mg/dL for men and 50 mg/dL for women; (ii) serum triglycerides >150 mg/dL; (iii) systolic blood pressure >130 mmHg or diastolic blood pressure >85 mmHg; or (iv) serum glucose >100 mg/dL. The MUOO group included individuals with BMI ≥25 kg/m^2 and presenting with at least one of the aforementioned cardiovascular risk factors.

Supervised exercise-intervention training program

The fully description of the supervised exercise training program can be found elsewhere (28). Briefly, the supervised exercise intervention combined endurance and resistance training, following the World Health Organization (WHO) guidelines. For 24 weeks, participants attended to the research center 3-4 times per week, and both endurance and resistance training were personalized to the participants' physical fitness levels. The intervention was divided in 5 phases of different durations, starting with a familiarization phase of 4 weeks (28). Participants completed 150min/week of endurance training, performed at 60% of heart rate reserve (HRR) in the Ex-MOD, whereas Ex-VIG performed 75min/week at 60% HRR and 75min/week at 80% HRR. Participants completed a total of 80min/week of resistance exercise, performed in 2 sessions/week, , performed with loads equivalent to 50%RM in the Ex-MOD and to 70%RM Ex-VIG. The load for resistance exercises were adjusted monthly (28). All exercise sessions were conducted in groups of 10-12 participants at the same time of the day during the whole intervention. Attendance was daily registered, and adherence to the prescribed intensity for the endurance training was quantified by heart rate monitors (RS800CX, Polar Electro Öy, Kempele, Finland). Participants were allowed and encouraged to perform unsupervised training sessions when they were unable to attend the research center.

Statistical analysis

Data are presented as means ± standard deviations (unless otherwise stated). Plasma succinate levels were computed as tertiles (low, intermediate or high levels) using the function "Visual Binning" with SPSS (SPSS v. 22.0, IBM SPSS Statistics, IBM Corp. Armonk, NY). For descriptive characteristics, categorical and continuous variables were used according to plasma succinate levels. Differences in categorical variables between groups were analyzed by chi-square tests, whereas differences in continuous variables between groups were analyzed by one-way analyses of variance. The level of significance between groups was set at $P<0.05$, after Bonferroni correction for multiple comparisons. Serum levels of cardiovascular risk parameters and plasma omega-3 and omega-6 oxylipins levels were log10 transformed to achieve a normal distribution. Plasma succinate levels followed a normal distribution and were not transformed. The plasma succinate fold-change value was used to perform interaction network pathway analyses of plasma omega-3 and omega-6 oxylipins, computed as the ratio of the mean values of the two compared groups (*i.e.*, high vs. low fold change = average succinate levels (high tertile)/ average succinate levels (low tertile). Fold-change

differences were analyzed with an unpaired t-test. The sex distribution in the classification of MHOO and MUOO individuals was not similar; therefore, to study whether plasma succinate levels were different between groups the analyses were adjusted by sex as a covariate. No sex interaction was detected in the other analyses (all P>0.05). **Fig. 3** and **Fig. S1** were built using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, CA). **Fig. 2** was built using Cytoscape software version 3.7.0 for Windows (Boston, MA) [67].

RESULTS

The characteristics of the participants included in the study are shown in **Table 2**. We found a great variability in plasma succinate levels in the cohort (11.6–129.8 μM; amplitude range, 118.2 μM) (**Fig. 1**). Given this broad range, we used the low (11.6–55.1 μM), intermediate (55.2–71.4 μM), and high (71.5–129.8 μM) plasma succinate tertiles for subsequent analyses (**Table 2**).

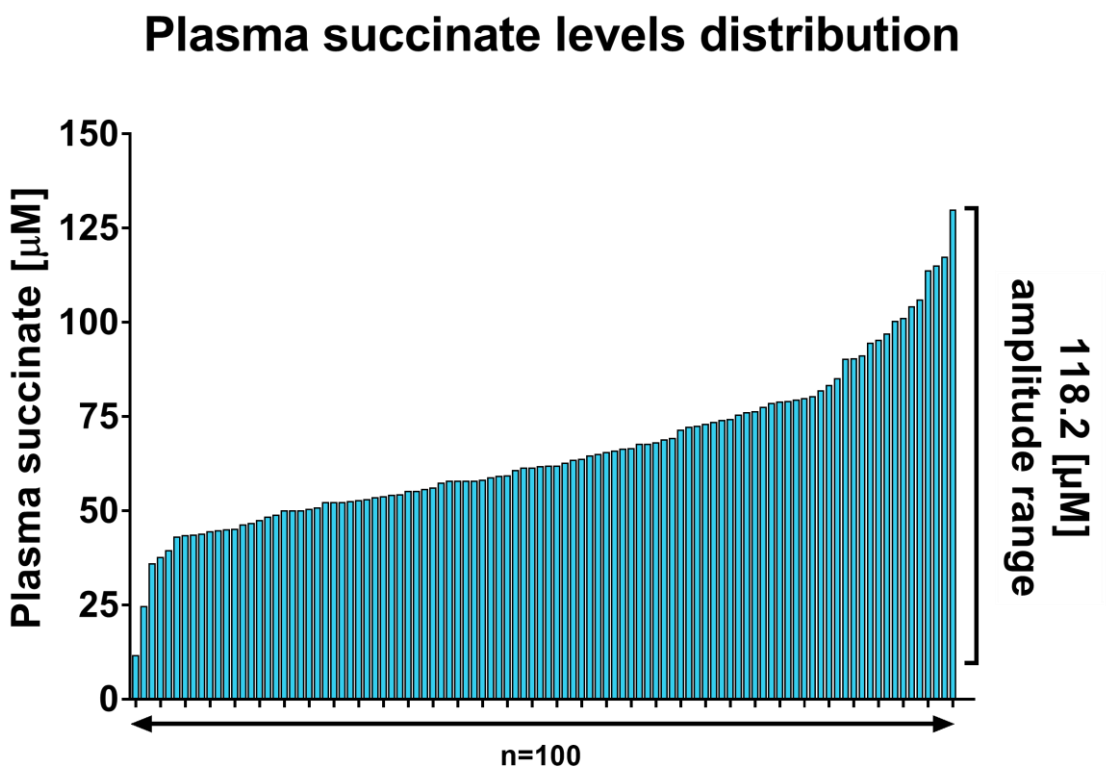


Figure 1. Waterfall plot showing plasma succinate levels per individual (n=100). Each bar represents a single individual.

Table 2. Characteristics of the individuals by tertiles of plasma succinate levels.

	Plasma succinate tertiles			
	Low (n=34)	Intermediate (n=33)	High (n=33)	
				P
Age (years)	21.7 ± 2.3	22.6 ± 2.1	21.6 ± 1.9	0.137
Sex (n,%)				0.777
Men	13 (38.2)	10 (30.3)	12 (36.4)	
Women	21 (61.8)	23 (69.7)	21 (64.6)	
Weight status (n,%)				0.097
Normal-weight	23 (67.7)	19 (57.6)	15 (45.5)	
Overweight	8 (23.5)	11 (33.3)	10 (30.3)	
Obese	3 (8.8)	3 (9.1)	8 (24.2)	
BMI (kg/m²)	24.2 ± 3.8	24.0 ± 3.8	26.1 ± 5.2	0.091
LMI (kg/m²)	14.8 ± 2.1	14.1 ± 2.2	15.1 ± 2.7	0.275
FMI (kg/m²)	8.1 ± 2.7	8.5 ± 2.6	9.6 ± 3.3	0.077
Body fat (%)	33.6 ± 7.4	35.8 ± 6.7	37.0 ± 8.1	0.170
VAT (g)	289* ± 146	346 ± 178	411* ± 201	**0.020**
Waist circumference (cm)	78.8 ± 11.9	80.9 ± 13.3	84.3 ± 14.7	0.247
Glucose (mg/dL)	86.5 ± 6.2	88.5 ± 6.5	87.3 ± 6.9	0.466
Insulin (µUI/mL)	7.4 ± 3.9	8.2 ± 3.9	9.6 ± 6.0	0.126
HOMA index	1.6 ± 1.0	1.8 ± 1.0	2.1 ± 1.6	0.152
Total cholesterol (mg/dL)	156.2 ± 25.0	162.6 ± 31	170.2 ± 34	0.230
HDL-C (mg/dL)	51.3 ± 9.7	52.4 ± 9.5	54.2 ± 15	0.808
LDL-C (mg/dL)	91.2 ± 23	94.3 ± 25	95.7 ± 29	0.907
Triglycerides (mg/dL)	68.2* ± 28	80.0 ± 40	111.8* ± 70	**0.002**
C-reactive protein (mg/L)	1.7* ± 2.2	2.1 ± 2.1	3.8* ± 5.1	**0.039**
SBP (mmHg)	114.1 ± 11	116.7 ± 11	120.4 ± 12	0.082
DBP (mmHg)	68.8* ± 7.7	71.4 ± 5.5	73.7* ± 7.6	**0.023**
Metabolic syndrome ATPIII (n,%)	0 (0)	1 (3)	4 (12.1)	0.068
Cardiorespiratory fitness (mL/kg/min)	44.9* ± 7.3	38.8* ± 7.7	40.6 ± 6.9	**0.004**
BAT volume (mL)	71.3 ± 47.3	71.0 ± 68.8	64.3 ± 56.2	0.825
BAT SUVmean	4.0 ± 2.1	3.6 ± 2.0	3.8 ± 1.8	0.794
Basal metabolic rate (kcal/d)	1335 ± 570	1518 ± 896	1407 ± 198	0.534
Energy intake (kcal/d)	1904 ± 463	1769 ± 397	1950 ± 497	0.115
Energy density intake (kcal/g/d)	1.5 ± 0.4	1.5 ± 0.3	1.4 ± 0.3	0.333
Fat intake (g/d)	84.8 ± 25.9	78.3 ± 26.4	85.7 ± 23.9	0.156
Protein intake (g/d)	77.8* ± 20.2	70.2* ± 14.6	76.4 ± 21.6	**0.003**
Carbohydrates intake (g/d)	202.4 ± 67.0	191.1 ± 64.4	214.2 ± 73.6	0.378

Data are presented as mean and standard deviation (SD), otherwise stated. P from one-way analysis of variance, or from chi-square test (categorical variables). Plasma succinate levels are computed as tertiles. *Symbols indicates significant differences between groups (P<0.05) after Bonferroni correction for multiple comparisons. ATPIII: National Cholesterol Education Program Adult Treatment Panel III; BAT: brown adipose tissue; BMI: body mass index; DBP: diastolic blood pressure; FMI: fat mass index; HDL-C: High density lipoprotein-cholesterol; HOMA index: homeostatic model assessment; LDL-C: Low density lipoprotein-cholesterol; LMI: lean mass index; SBP: systolic blood pressure; SUV: standardized uptake value; VAT: visceral adipose tissue.

Young adults with higher plasma succinate levels have higher visceral adiposity and an adverse cardiovascular profile

Participants in the highest tertile of succinate had significantly higher visceral adipose tissue (VAT) mass (+42.5%), serum triglyceride levels (+63.9%), serum C-reactive protein levels (+124.2%), and diastolic blood pressure (+5.5%) than peers in the lowest tertile (**Table 2**). By contrast, cardiorespiratory fitness levels were significantly higher in the lowest tertile (up to +15.7%) than in the intermediate tertile (**Table 2**). No significant differences were found in dietary energy and macronutrients intake, dietary energy density parameters, BMR levels or BAT parameters across the three tertiles (**Table 2**).

Plasma succinate levels are not associated with fecal microbiota composition and diversity

Analysis of the fecal microbiota in young adults revealed no association between succinate levels and beta or alpha diversity (all P≥0.380 **Fig. 2a, b)**. Similarly, no associations were found between succinate levels and relative abundances at the phylum level (all P>0.05; **Fig. 2c, left panel).** Nonetheless, we found that individuals in the lowest tertile of plasma succinate had a higher relative abundance of *Bacteroides* (+56.9%) and a lower relative abundance of *Acidaminococcus* (-93.8%) than individuals in the intermediate and highest tertiles, respectively (all P≤0.01; **Fig. 2c, right panel).** No significant differences were observed in the relative abundance of the species belonging to *Bacteroides* and *Acidaminococcus* genera across the succinate tertiles (all P>0.05; **Table 3**). Likewise, no associations were found between succinate levels and succinate-producing or succinate-consuming species (all P>0.05; **Table 4** and **Fig. 3a, b**).

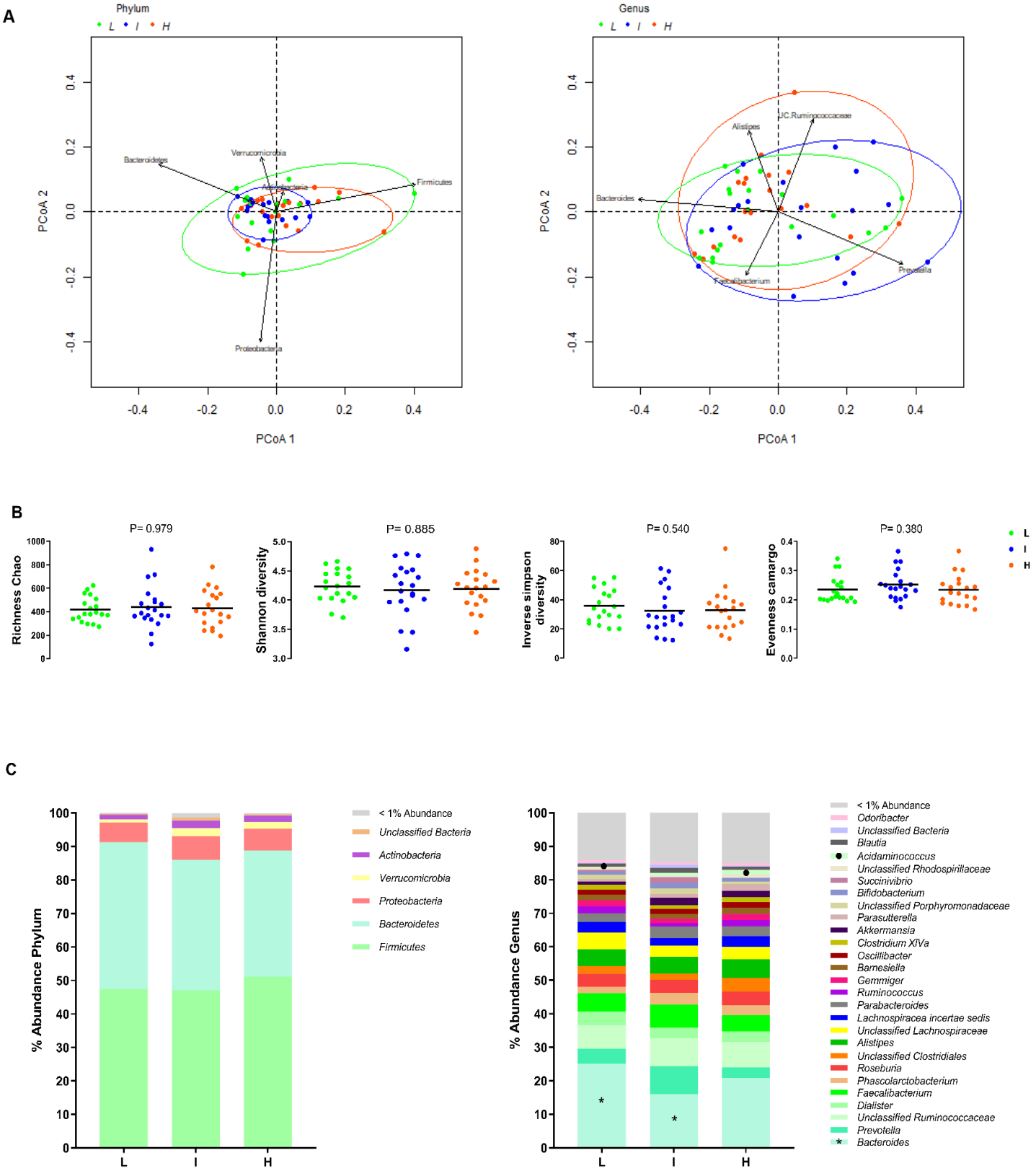


Fig. 2. Fecal microbiota diversity and composition by tertiles of plasma succinate (n=58). L: Low succinate plasma concentration (11.6–57.3 µM); I: Intermediate succinate plasma concentration (57.3–75.3 µM); H: High succinate plasma concentration (75.4–129.8 µM). A) Principal Coordinate Analysis (PCoA) plot of the first two principal coordinates at phylum and genus level, categorized by circulating succinate levels. Genus PCoA only shows PCoA analyses done using Bray-Curtis dissimilarity. Ellipses represent the 95% confidence intervals (package, vegan, R version 3.6). B) Differences between the circulating succinate tertiles in fecal microbiota diversity indexes (richness Chao, Shannon, inverse of Simpson, and evenness Camargo). Kruskal-Wallis test (P<0.05) was used to test for each pairwise comparison. C) Relative abundance of the fecal microbiota at phylum (left panel) and genus level (right panel) according to circulating succinate levels. Stacked bar represented percentage abundance. The symbol (*) means statistical significance differences between Low and Intermediate levels, whereas the symbol (•) means statistical significance differences between Low and High levels, determined by Kruskal-Wallis test, corrected for multiple comparisons FDR (P<0.05).

Table 3. Relative abundance (%) of species belonging to the *Bacteroides* and *Acidaminococcus* genera by tertiles of plasma succinate (n=58).

	Plasma succinate tertiles		
	Low (11.6–57.3 µM) n=19	Intermediate (57.8–75.4 µM) n=20	High (76.3–129.8 µM) n=19	
Bacteroides genus (%)				P
Bacteroides caccae	0.62 ± 1.26	0.40 ± 0.50	0.66 ± 1.10	0.729
Bacteroides cellulosilyticus	0.70 ± 1.78	0.15 ± 0.23	0.42 ± 0.59	0.776
Bacteroides clarus	0.00 ± 0.00	0.04 ± 0.08	0.03 ± 0.07	0.099
Bacteroides coprocola	1.25 ± 2.36	1.79 ± 3.21	0.26 ± 0.84	0.264
Bacteroides coprophilus	0.01 ± 0.03	0.32 ± 1.43	0.11 ± 0.50	0.462
Bacteroides dorei	3.33 ± 4.54	2.57 ± 3.79	4.69 ± 5.40	0.449
Bacteroides eggerthii	0.38 ± 1.42	0.09 ± 0.18	0.41 ± 0.81	0.730
Bacteroides faecis	0.32 ± 0.58	0.21 ± 0.41	0.10 ± 0.24	0.176
Bacteroides finegoldii	0.32 ± 1.06	0.04 ± 0.10	0.16 ± 0.41	0.796
Bacteroides fragilis	0.66 ± 1.65	0.15 ± 0.37	0.29 ± 0.70	0.408
Bacteroides intestinalis	0.02 ± 0.09	0.01 ± 0.02	0.01 ± 0.02	0.435
Bacteroides massiliensis	1.40 ± 2.67	1.00 ± 1.42	0.87 ± 1.36	0.853
Bacteroides nordii	0.00 ± 0.01	0.01 ± 0.02	0.00 ± 0.01	0.692
Bacteroides ovatus	0.45 ± 0.85	0.44 ± 1.17	0.15 ± 0.14	0.537
Bacteroides plebeius	0.54 ± 0.92	0.45 ± 1.63	0.16 ± 0.38	0.696
Bacteroides salyersiae	0.10 ± 0.38	0.02 ± 0.07	0.00 ± 0.01	0.635
Bacteroides sartorii	0.00 ± 0.00	0.02 ± 0.10	0.00 ± 0.00	0.387
Bacteroides stercoris	0.38 ± 1.01	1.05 ± 3.55	0.14 ± 0.32	0.741
Bacteroides thetaiotaomicron	0.14 ± 0.21	0.18 ± 0.28	0.50 ± 0.86	0.139
Bacteroides uniformis	3.37 ± 2.84	2.34 ± 3.01	3.80 ± 3.15	0.209
Bacteroides vulgatus	6.94 ± 7.06	2.42 ± 2.67	4.90 ± 5.56	0.186
Bacteroides xylanisolvens	0.44 ± 0.62	0.27 ± 0.41	0.58 ± 0.83	0.234
Acidaminococcus genus (%)				
Acidaminococcus fermentans	0.00 ± 0.00	0.00 ± 0.00	0.33 ± 1.43	0.358
Acidaminococcus intestini	0.08 ± 0.26	0.92 ± 1.80	0.89 ± 1.54	0.086

Data are presented as mean and standard deviation (SD). P-value from the Kruskal-Wallis test, correcting for multiple comparisons FDR (P≤0.05).

Table 4. Relative abundance (%) of succinate-producing and -consuming species previously described by Serena C. et al. [23] by tertiles of plasma succinate (n=58).

	Plasma succinate tertiles		
	Low (11.6–57.3 µM) n=19	Intermediate (57.8–75.4 µM) n=20	High (76.3–129.8 µM) n=19	
Succinate-producers (%)				P
Bacteroides fragilis	0.66 ± 1.65	0.15 ± 0.37	0.29 ± 0.70	0.408
Bacteroides vulgatus	6.94 ± 7.06	2.42 ± 2.67	4.90 ± 5.56	0.186
Parabacteroides distasonis	0.36 ± 0.56	1.45 ± 4.48	0.46 ± 0.76	0.491
Paraprevotella xylaniphila	0.34 ± 1.48	0.08 ± 0.28	0.08 ± 0.33	0.606
Alistipes indistinctus	0.07 ± 0.13	0.18 ± 0.42	0.11 ± 0.22	0.467
Blautia wexlerae	0.31 ± 0.36	0.71 ± 1.85	0.32 ± 0.52	0.437
Faecalibacterium prausnitzii	2.40 ± 1.38	3.03 ± 2.75	1.93 ± 1.68	0.298
Akkermansia muciniphila	0.76 ± 1.34	2.28 ± 4.34	1.53 ± 2.49	0.321
Succinate-consumers (%)				
Bacteroides thetaiotaomicron	0.14 ± 0.21	0.18 ± 0.28	0.50 ± 0.86	0.139
Phascolarctobacterium faecium	1.39 ± 2.94	1.85 ± 3.82	2.54 ± 5.10	0.609
Phascolarctobacterium succinatutens	0.47 ± 1.42	0.79 ± 1.53	0.11 ± 0.42	0.712
Ruminococcus bromii	0.17 ± 0.41	0.42 ± 1.03	0.42 ± 1.34	0.738
Dialister propionicifaciens	0.03 ± 0.11	0.00 ± 0.01	0.00 ± 0.00	0.645
Dialister succinatiphilus	0.00 ± 0.00	0.00 ± 0.01	0.65 ± 2.00	0.354

Data are presented as mean and standard deviation (SD). P-value from the Kruskal-Wallis test, correcting for multiple comparisons FDR (P≤0.05).

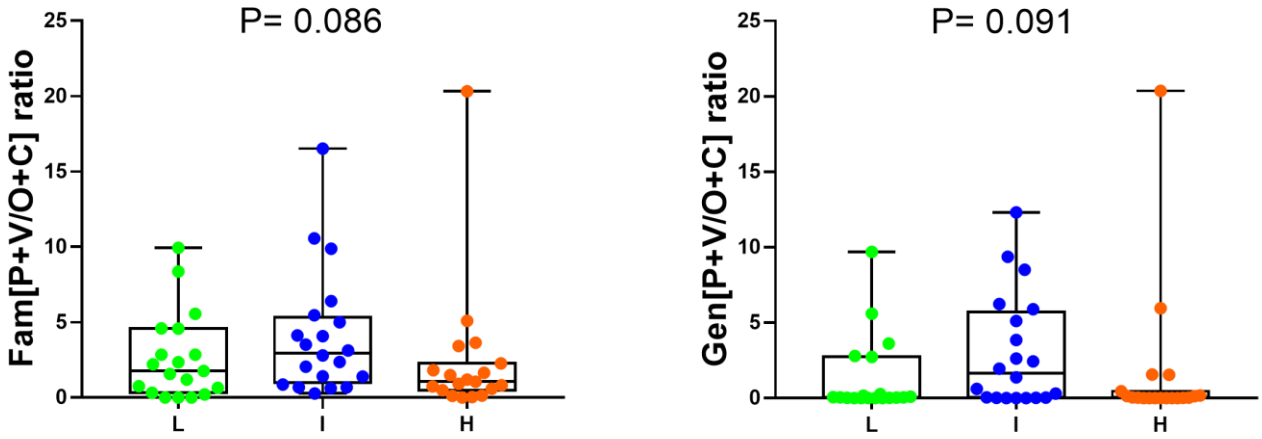


Figure 3. Differences at family (A) and genus (B) levels by tertiles of plasma succinate levels (n=58): families (*Prevotellaceae* plus *Veillonellaceae/Odoribacteriaceae* plus *Clostridaceae*) [fam(P +V/O + C)] ratio; genera (*Prevotellaceae spp.* plus *Veillonellaceae spp./Odoribacteriaceae spp.* plus *Clostridaceae spp.*) [gen(P +V/O + C)] ratio. P-value from the Kruskal-Wallis test, correcting for multiple comparisons FDR (P<0.05).

Plasma succinate levels are associated with pro-inflammatory omega-6 oxylipins

Given the clear link between plasma succinate and some pro-inflammatory markers, such as serum C-reactive protein and serum triglycerides (**Table 2**) [68], and the lack of associations between plasma succinate levels and classical inflammatory markers (*i.e.*, L-6, TNF-α or IFN-γ; data not shown), we extended our investigation to the fatty acid-derived oxylipins. No significant differences were observed in plasma omega-3 oxylipins when comparing the high *versus* low succinate tertiles (**Fig. 4a**) (abbreviations are detailed in **Table 1**). However, individuals in the highest succinate tertile had significantly higher plasma concentrations of omega-6 oxylipins than individuals in the lowest tertile, including the omega-6 fatty acids DGLA (+61.1%), AdrA (+47.4%) and AA (+28.7%), as well as several of their downstream products, as revealed by interaction network pathway analysis (**Fig. 4b**). In addition, individuals in the highest succinate tertile had significantly lower levels of the omega-6 oxylipins 12,13-EpOME (-30.7%) and 1a,1b-dihomo PGF_{2alpha} (-25.4%). With respect to DGLA metabolism, the hydroxy-trienoic acid product resulting from 15-lipoxygenation of DGLA (15-HETrE), was found to be significantly higher in concentration in individuals in the highest succinate tertile than in those in the lowest succinate tertile (+21.8%). The concentration of other downstream AA-derived oxylipins was also significantly higher in individuals in the highest tertile of plasma succinate, including 11-HETE (+20.4%), 12-HETE (+74.9%), 12-HHTrE (+97.9%) and TxB2 (+84.8%).

Only small differences in oxylipin levels were observed when comparing high *versus* medium succinate tertiles (**Fig. 5A**), but the differences observed between medium *versus* low succinate tertiles (**Fig. S5B**) resembled the differences between high and low succinate tertiles.

High vs. Low succinate levels

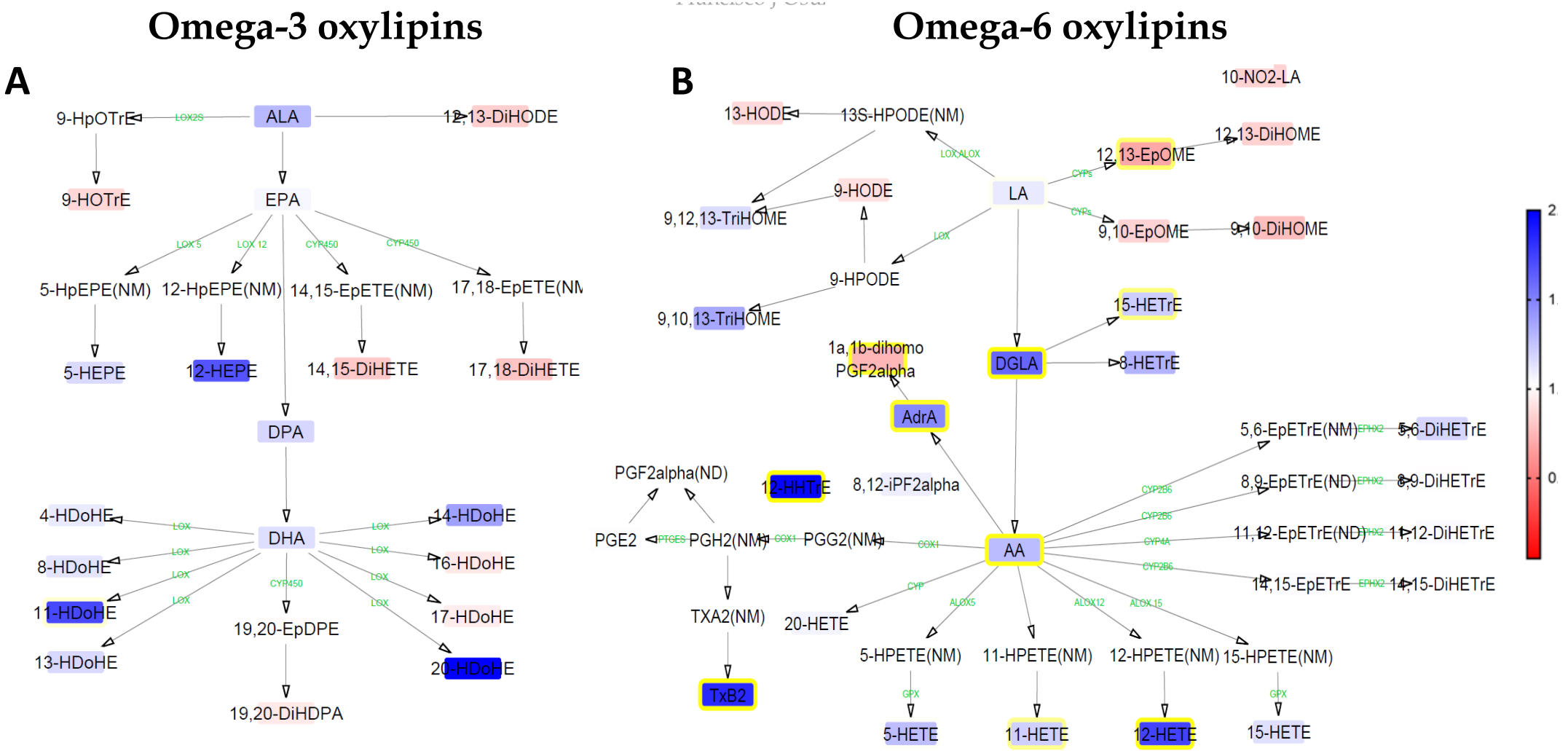
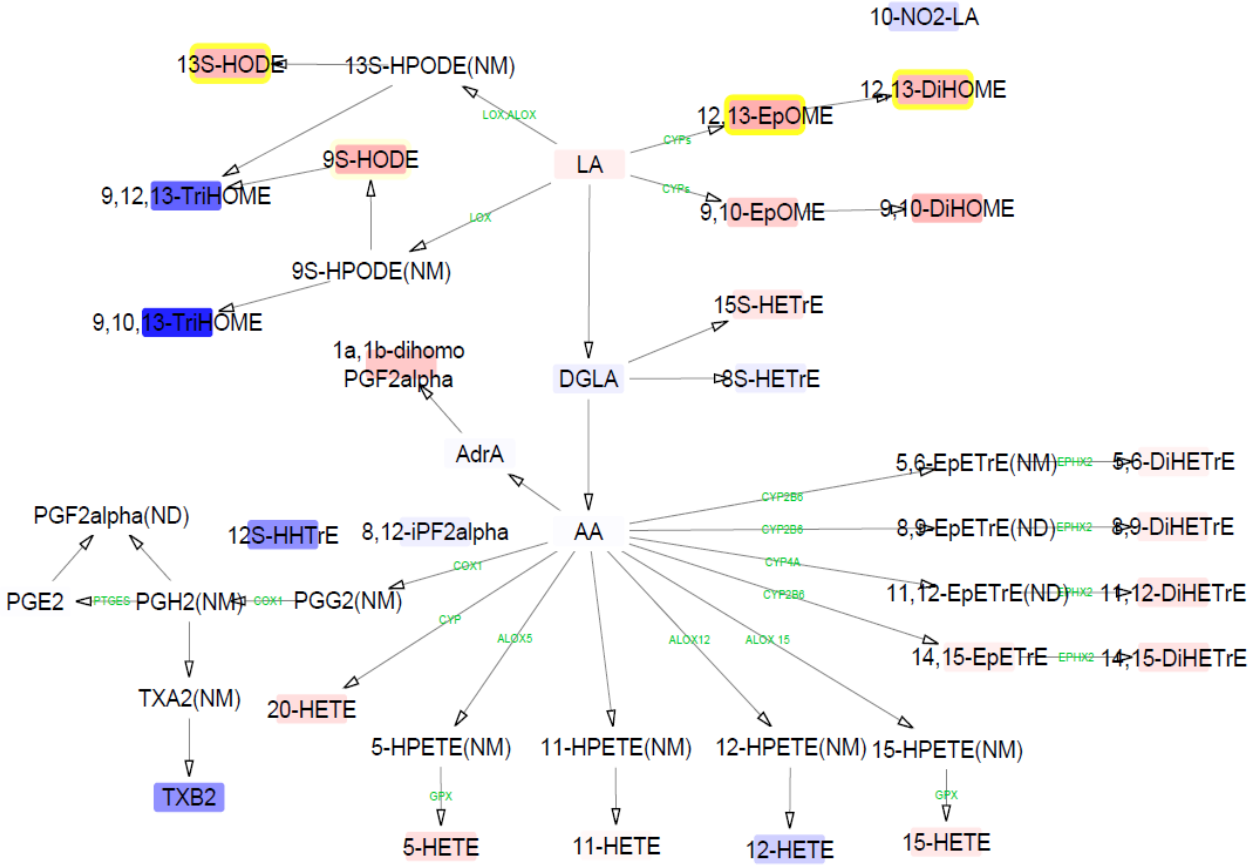


Fig. 4. Interaction network pathway analysis of circulating omega-3 and omega-6 oxylipins (n=98). The networks depict the differences of each lipid mediator between high *versus* low tertiles of plasma succinate levels. Compounds that were not detected (ND) or not measured (NM) with the LC-MS/MS method are shown without boxes. Blue boxes with yellow borderlines indicate that this lipid mediator was higher in high *versus* the low succinate group, whereas red boxes with yellow borderlines indicate that this lipid mediator was lower in the comparison. Boxes without yellow borderlines indicate that this lipid mediator did not significantly change in the comparison. Fold-change values are depicted according to the legend bar on the right side. Intermediate enzymes are represented in green, and they were not measured. Comparisons are performed with independent t-test analysis (log10 transformed values) and P<0.05.

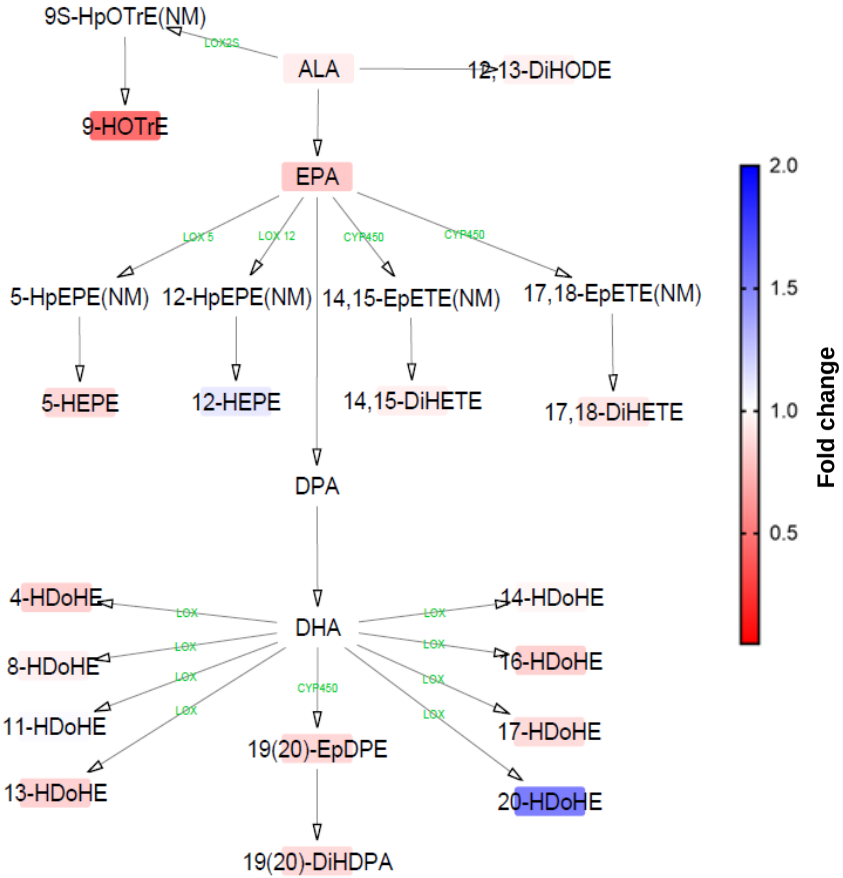
A

High vs. Medium

Omega-6 oxylipins



Omega-3 oxylipins



Medium vs. Low

Omega-6

Omega-3

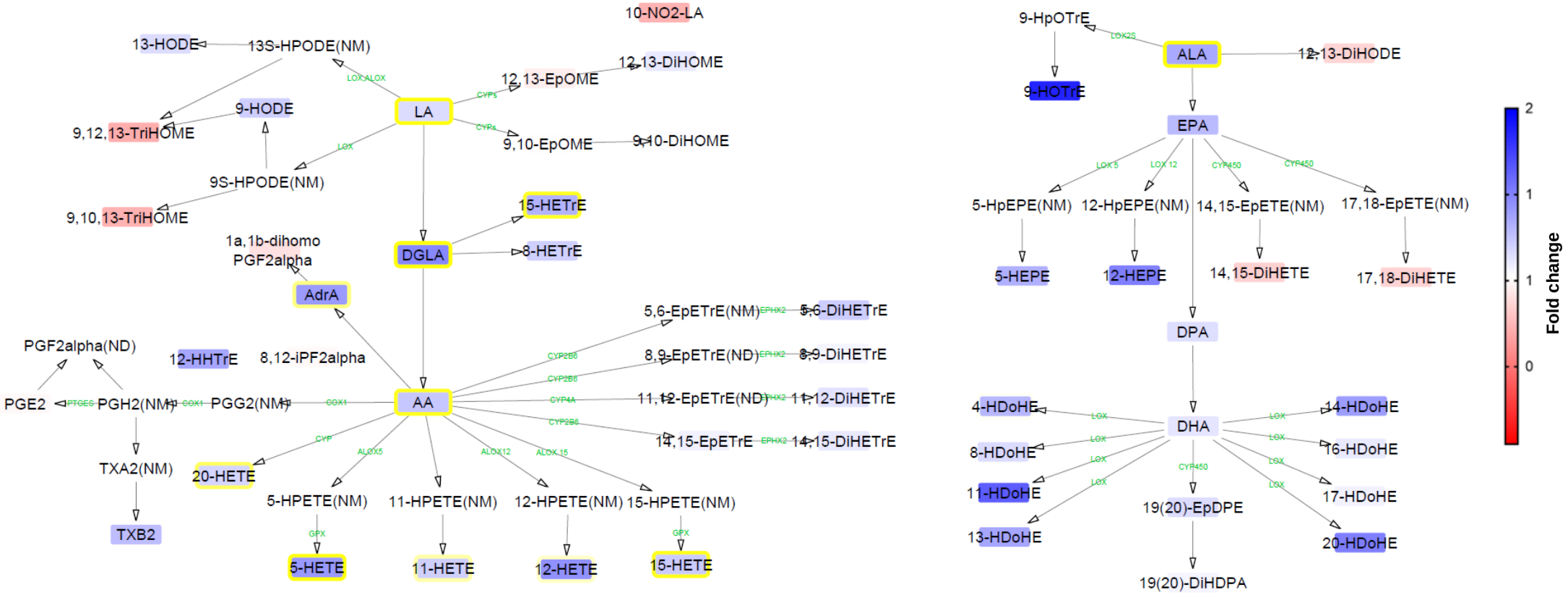


Fig. 5. Interaction network pathway analysis of circulating omega-3 and omega-6 oxylipins (n=98). The networks depict the differences of each lipid mediator between high versus medium tertiles of plasma succinate levels (A) and medium versus low tertiles of plasma succinate levels (B). Compounds that were not detected (ND) or not measured (NM) with the LC-MS/MS method are shown without boxes. Blue boxes with yellow borderlines indicate that this lipid mediator was higher in high versus the low succinate group, whereas red boxes with yellow borderlines indicate that this lipid mediator was lower in the comparison. Boxes without yellow borderlines indicate that this lipid mediator did not significantly change in the comparison. Fold-change values are depicted according to the legend bar on the right side. Intermediate enzymes are represented in green and they were not measured. Comparisons are performed with independent t-test analysis (log10 transformed values) and P<0.05.

Young adults with metabolically unhealthy overweight/obesity have higher plasma succinate levels than their metabolically healthy counterparts

To gain more insight into the potential role of plasma succinate as an early marker of cardiovascular risk, we subcategorized the overweight/obese individuals of the cohort (43% of our population) as healthy (MHOO; n=27) or unhealthy (MUOO; n=16) based on their cardiovascular profile. Individuals in the MUOO group had higher BMI (+7.2%), FMI (+3.1%), VAT mass (+24.3%), fasting glucose (+7.6%), insulin (+57.9%), homeostatic model assessment index (+71.5%), total cholesterol (+15.4%), triglycerides (+119.8%), and systolic (+8.2%) and diastolic (+10.1%) blood pressure than peers in the MHOO group, and had lower HDL-C levels (-16.1%) (**Table 5**). Moreover, plasma succinate levels were significantly higher (+21.3%) in the MUOO group (75.5 ± 12.3 μM) than in the MHOO group (62.3 ± 17.4 μM) (P=0.009; **Fig. 6**).

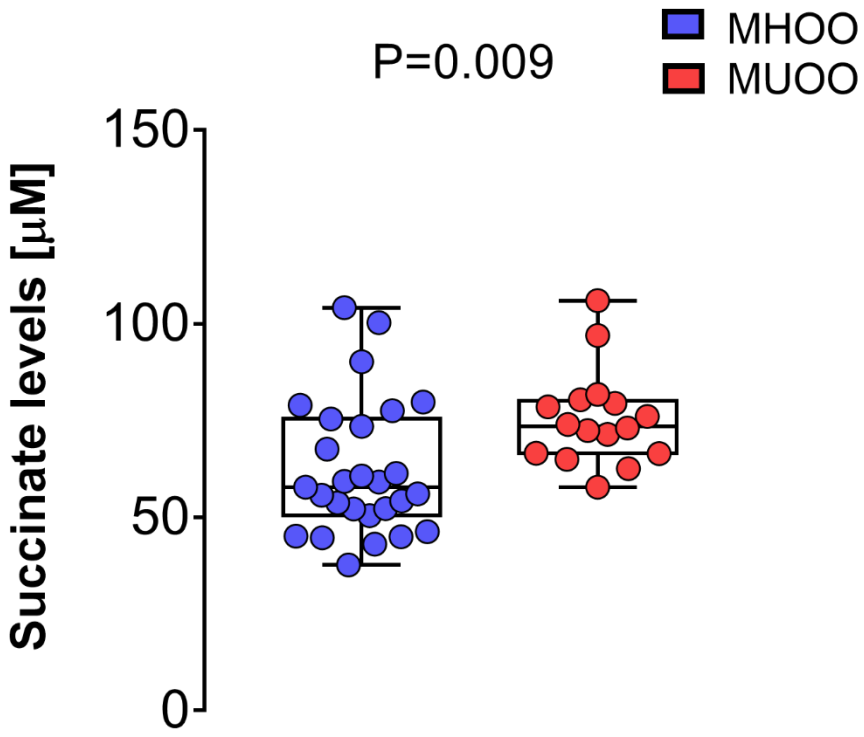


Figure 5. Comparisons between plasma succinate levels in metabolic healthy overweight/obese (MHOO, n=27) and metabolic unhealthy overweight/obese (MUOO, n=16) young adults. P value obtained from one-way analysis of variance adjusted for sex.

Table 5. Characteristics of metabolically healthy overweight/obese (MHOO, n=27) and metabolically unhealthy overweight/obese (MUOO, n=16) individuals

	MHOO	MUOO	P
Sex (n,%)			**0.013**
Men	8 (29.6)	11 (68.8)	
Women	19 (70.4)	5 (31.2)	
Age (years)	21.9 ± 2.4	22.7 ± 2.5	0.663
BMI (kg/m^2)	28.2 ± 2.4	30.3 ± 3.1	0.050
LMI (kg/m^2)	15.7 ± 2	17.3 ± 1.9	0.376
FMI (kg/m^2)	11.1 ± 2.1	11.4 ± 2.2	**0.023**
Body fat (%)	40.1 ± 6.6	38.5 ± 5.3	0.071
VAT (g)	459 ± 147	570.6 ± 127	**0.036**
Glucose (mg/dL)	86.9 ± 6.6	93.6 ± 6.9	**0.004**
Insulin (µUl/mL)	8.7 ± 3.9	13.8 ± 7.2	**0.001**
HOMA index	1.9 ± 1	3.3 ± 1.9	**0.001**
Total cholesterol (mg/dL)	152.1 ± 21.7	175.6 ± 38	**0.035**
HDL-C (mg/dL)	50.6 ± 9.3	42.5 ± 9.3	**0.035**
LDL-C (mg/dL)	88 ± 16.9	107.6 ± 36	0.161
Triglycerides (mg/dL)	67.6 ± 23.7	148.6 ± 86	**<0.001**
C-reactive protein (mg/L)	2.7 ± 2.3	4.7 ± 6.3	0.203
SBP (mmHg)	117.5 ± 7.5	129.3 ± 10	**0.003**
DBP (mmHg)	71.4 ± 6.01	77.8 ± 7.3	**0.016**
Metabolic syndrome ATPIII (n,%)	0 (0)	5 (31)	**0.003**
Cardiorespiratory fitness (mL/kg/min)	38.3 ± 8.3	37.7 ± 6.4	**0.048**

Data are presented as mean and standard deviation (SD), unless stated otherwise. P-value from one-way analysis of variance, which was conducted with log10 transformed data and sex adjusted, or from chi-square for categorical variables. ATP III: National Cholesterol Education Program Adult Treatment Panel III. BMI: body mass index; DBP: diastolic blood pressure; FMI: fat mass index; HDL-C: High-density lipoprotein cholesterol; HOMA: homeostatic model assessment; LDL-C: Low-density lipoprotein cholesterol; LMI: lean mass index; MHOO: metabolically healthy overweight-obese; MUOO: metabolically unhealthy overweight-obese; SBP: systolic blood pressure; VAT: visceral adipose tissue.

A 24-weeks exercise-intervention training program does not modify plasma succinate levels

Of the 100 participants that started the exercise program, 99 had valid succinate determinations after the 24 weeks intervention. Of them, 12 participants from the EX-MOD group and 11 participants from the EX-VIG group were excluded from the main analyses for attending less than 70% of the total training sessions. 24 weeks of supervise exercise training does not modify plasma succinate levels [Δ mean and standard deviation: Δ CON= 4.1 ± 22.8; Δ EX-MOD= 4.3 ± 21.8; Δ EX-VIG= -1.8 ± 19.9, **Fig. 7**. The results were not altered when other attendance criteria (i.e., <70% or ≥85%) were applied (data not shown).

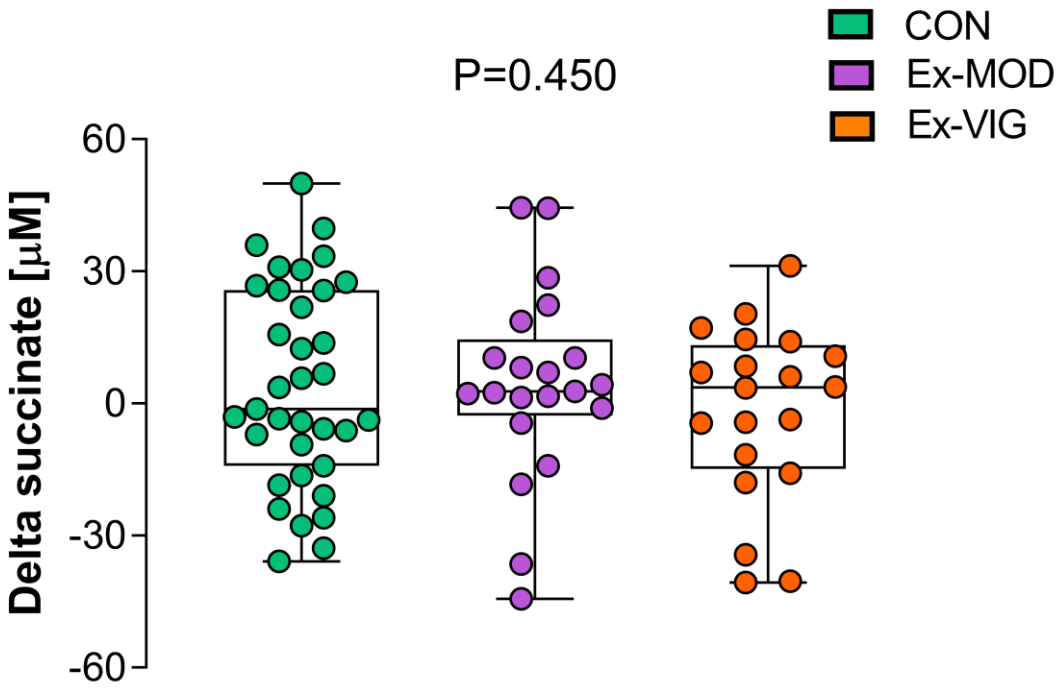


Figure 7. Effects of a 24 weeks of exercise training program on plasma succinate levels in young adults. CON (n=35), Ex-MOD (n=21), Ex-VIG (n=20).

DISCUSSION

Here, we demonstrate for the first time to our knowledge that plasma succinate is associated with VAT mass, serum triglycerides and C-reactive protein levels, and diastolic blood pressure in young adults. Likewise, individuals with higher levels of plasma succinate have higher levels of plasma omega-6 oxylipins, which are linked to increased pro-inflammatory status and, accordingly, elevated CVD risk [31,32]. By contrast, plasma succinate levels are not associated with BAT or with fecal microbiota composition and diversity. Interestingly, individuals who are metabolically unhealthy with overweight/obesity have higher plasma succinate levels than their metabolically healthy counterparts. However, plasma succinate levels are not modified after 24-weeks of an exercise-intervention program. Collectively, our findings suggest that plasma succinate is candidate biomarker of cardiovascular risk in young adults. Further studies are, nevertheless, needed to unravel the underlying mechanisms that may explain these associations.

Investigations of the association of plasma succinate levels with body composition are scarce. We recently showed that plasma succinate levels are positively associated with BMI in middle-aged and elderly adults with obesity and T2D [24,26]. In the present study, we found that young adults with higher

succinate levels have higher VAT mass, an established marker of elevated CVD risk [69–71]. Visceral fat depots are linked to metabolic dysfunction through increased mitochondrial oxidative stress, a main driver of cellular insulin resistance [72]. Interestingly, succinate dehydrogenase (SDH) activity is a potential source of reactive oxygen species (ROS) under specific conditions, including obesity [73–75], suggesting an adverse influence of SDH in obesity. In this context, some of the metabolic improvements observed after bariatric surgery are, in part, due to the restoration of SDH activity in VAT, which concurs with the weight loss caused by the surgery [76]. It is thus reasonable to propose that a fraction of the plasma succinate could originate in (and be secreted from) VAT depots, through SDH activity, contributing to the positive association between plasma succinate levels and VAT mass.

The gut microbiota plays a key role in regulating host metabolism, and specific signatures of gut microbiota composition have been associated with obesity, insulin resistance, and T2D [77]. Succinate is a primary cross-feeding metabolite between gut-resident microbes, which is important to preserve a healthy gut microbiota [23]. We recently demonstrated in middle-aged adults that the fecal microbiota is a putative contributor of circulating succinate levels in some health conditions such as obesity [26]. Our previous data also support the notion that succinate is a marker of microbiota dysbiosis, as intestinal permeability positively correlates with circulating succinate in individuals with obesity [26]. Nonetheless, we found no association between plasma succinate and succinate-producing or succinate-consuming bacteria species in the present analysis. Differences in the age and metabolic status of the cohorts, or even the moderate sample size of the present study (n=100), may partly explain these findings.

We previously showed that circulating succinate is regulated nutritionally [22], and it is known that SUCNR1 has an intracellular anti-lipolytic function [78,79]. Studies in mice have recently demonstrated that succinate uptake stimulates uncoupling protein 1 (UCP1)-dependent thermogenesis in brown adipocytes *via* ROS production, protecting against diet-induced [19]. Our present study, however, failed to show a significant association between systemic succinate and BAT volume or activity. Given the cross-sectional nature of our study, we cannot draw conclusions on a potential role of succinate in human BAT thermogenesis. Additionally, neither plasma succinate levels nor BMR were associated with parameters of energy intake/consumption in our young cohort. Nonetheless, plasma succinate levels were inversely associated with cardiorespiratory fitness. In this line, succinate has recently emerged as an important player in muscle

adaptation in response to exercise [20,80]. While the observational design of our present study does not enable us to infer causality, it adds to the increasing body of evidence on the role of succinate as a possible mediator of the cardiovascular benefits of exercise. Cardiorespiratory fitness is also recognized as a relevant risk factor associated with adverse cardiovascular health and poor prognosis [81], and a lower prevalence of metabolic syndrome has been reported in people with better cardiorespiratory fitness [82]. This may fit with our finding that the MUOO group had significantly higher succinate levels than the MHOO group, which also supports previous data showing higher succinate levels in obese individuals with T2D than in their healthy obese counterparts [26]. Altogether, these results strengthen the hypothesis that high plasma succinate levels are linked to an impaired metabolic status.

Young adults with high plasma succinate levels also had higher plasma levels of C-reactive protein and pro-inflammatory oxylipins. Omega-6 oxylipins are key metabolites in pro-inflammatory processes and are closely linked to the progression of obesity and to cardiovascular risk [31,32], whereas omega-3 oxylipins usually have opposing effects [31,32]. Specifically, we observed that circulating succinate levels were related to the omega-6 oxylipins 11-HETE, 12-HETE, 12-HHTrE, and 15-HETrE. Previous research has linked 5-HETE and 11-HETE to obesity [83–85], whereas the auto-oxidative product 15-HETrE has been associated with a high risk of cardiovascular events [86]. Of particular note is the high level of TxB2 in individuals with high succinate, which is reported to increase in patients with coronary atherosclerosis [87]. In addition to an increase in AA products in individuals with high succinate levels, we also observed an increase in the precursor DGLA, which has been previously associated with obesity and insulin resistance [88]. Likewise, the AA metabolic product AdrA, which was higher among participants in the highest succinate tertile, has been shown to be directly associated with the risk of all-cause mortality [89]. As many of the enzymes (*i.e.*, cyclooxygenases and lipoxygenases) involved in oxylipin metabolism are shared by both omega-3 and omega-6 oxylipins [90], it is not surprising to find an asymmetric pattern in the omega-3 and omega-6 oxylipins profile in the high *versus* low succinate groups.

Recently, an elegant study showed that muscle cells release succinate through a pH-Gated mechanism that involves the membrane transporter monocarboxylate transporter 1 (MCT1) during exercise [20]. These increases in circulating succinate levels are liked to muscle adaptations and remodeling via SUCNR1 activation [20]. To the best of our knowledge, no study has evaluated the effects of exercise training on circulating succinate levels in humans. We found

that 24-weeks of exercise training did not modify plasma succinate levels in young adults. Nevertheless, this is not a surprising finding since other traditional cardiometabolic risk factors that were either associated (i.e., serum triglycerides, C-reactive protein, or blood pressure levels) or not associated (i.e., serum glucose, insulin, total cholesterol, HDL-C, or LDL-C) with plasma succinate levels were not affected after 24-weeks of exercise (data not shown). Actually, these findings concur with previous exercise intervention studies conducted in relatively healthy individuals in which these circulating cardiometabolic risk markers were not altered by after exercise interventions [91–93]. Thus, the absence of exercise effect on cardiometabolic risk factors could be explained by the young age (22±2 years old) and relatively healthy status of our participants whose cardiometabolic risk markers were within normal ranges. Further studies with different types of exercise interventions and duration are needed to confirm these results.

Strengths and limitations

A major strength of the present study is the well-characterized population, including detailed measurements of body composition, BAT, and novel markers of the cardiometabolic profile such as oxylipins, which allow us to gain new insight into the inflammatory status of the individuals compared with classical inflammatory markers (*i.e.*, IL-6, TNF-α or IFN-γ). A major limitation of the study is its cross-sectional design, and no causality can be established. Another limitation is the limited sample size for multivariate statistical analyses. Finally, although ^{18}F-FDG uptake is the current gold-standard for BAT quantification, it also has limitations in the assessment of BAT metabolic activity and volume [94].

CONCLUSION

In conclusion, our study reveals that plasma succinate levels are linked to a specific pro-inflammatory omega-6 signature pattern and higher VAT levels, and might be useful as a novel clinical tool to identify young individuals at higher CVD risk, allowing the implementation of effective preventive treatment. Plasma levels of succinate seem to reflect the cardiovascular status of young adults, supporting its potential as a biomarker of CVD risk. However, plasma succinate levels are not modified after a 24-weeks exercise-intervention program. Prospective studies are needed to confirm its clinical relevance and predictive value as a CVD risk biomarker, an whether other type of exercise interventions programs could actually modify plasma succinate levels.

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RESULTS AND DISCUSSION

SECTION I

STUDY II: A single bout of aerobic and resistance exercise rapidly decreases plasma levels of bile acids in a different manner in young, sedentary adults: role of physical fitness

(In preparation)

ABSTRACT

Background: Acute exercise elicits complex changes in the concentration of circulating molecules which are partially modulated by the metabolic demands of contracting muscle. Circulating bile acids (BA) are signaling molecules that control energy expenditure, glucose, and lipid metabolism. However, the effects of acute exercise on plasma levels of BA in humans remains unexplored.

Objective: To investigate the effects of an acute maximal endurance exercise (EE) and resistance exercise (RE) on plasma levels of BA in young adults. Additionally, we investigated whether these effects were different depending on the individual's fitness levels.

Methods: Cardiorespiratory fitness (CRF) was assessed through a maximum walking effort test in 14 young adults (21.8 ± 2.5 yr., 12 women); whereas muscle strength in lower major muscle groups was assessed through resistance exercises in 17 young adults (22.4 ± 2.5 yr., 11 women). The concentration of 8 plasma BA were measured with liquid chromatography – tandem mass spectrometry before and, 3, 30, 60, and 120 min after each exercise bout. Body composition, serum cardiometabolic risk factors, brown adipose tissue, and fecal microbiota composition (16S rRNA sequencing) were measured.

Results: EE acutely and transiently decreased the plasma levels of total, primary and secondary BA ($P \leq 0.014$) just after exercise, and in the 30 min after exercise, followed by an overall increase that was more pronounced in the case of primary BA. Acute RE exerted a rapid and more prolonged reduction of plasma levels of secondary BA that lasted until 120 min after exercise ($P < 0.001$). The kinetics of plasma levels of primary BA, cholic acid (CA) and chenodeoxycholic acid (CDCA) after EE were different across individuals with low and high CRF levels ($P \leq 0.044$). Contrary, there were no differences on plasma levels of BA after RE between individuals with low and high levels of lower and upper body muscular strength ($P > 0.05$). Those individuals with high CRF levels presented increased plasma levels of CA and CDCA at 120 min as well as an improved glucose and immuno-metabolic profile in comparison with the low CRF group. The fecal microbiota diversity and composition was similar between individuals with high vs. low CRF levels.

Conclusion: Overall, we found that EE and RE acutely and transiently reduces plasma levels of BA in an exercise-type specific manner. Individuals with high CRF levels display an increase in plasma levels of CA and CDCA after 120 min of EE that seem to be reflective of their better health status vs. their low CRF levels counterparts

BACKGROUND

Acute exercise elicits a complex metabolic response that involves a complex organ and cellular communication through changes in the concentration of a myriad of molecules and metabolites [1]. A better comprehension of this response will help to unravel the mechanism by which cellular and biochemical pathways are affected by exercise. For these reasons, the implementation of new molecular techniques in the exercise physiology field, such as metabolomics and lipidomics, are providing new insights into the metabolic and molecular pathways involved in the health-related benefits of exercise [2,3].

The liver is an organ with major implications in the regulation of energy metabolism, and it is the site of production of bile acids (BA) from cholesterol [4]. Primary BA cholic (CA) and chenodeoxycholic acid (CDCA) are synthesized in the hepatocytes and then conjugated either with glycine (~75%) or taurine (~25%) [5]. Next, they are stored in the gallbladder and secreted within the bile to the duodenum, helping to the absorption of dietary lipids and fat-soluble vitamins [6]. The primary BA that reach the colon can undergo metabolic conversions by certain gut microbiota bacteria that expresses enzymes involved in BA metabolism to transform primary to secondary BA, such as deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA) [5]. Approximately 90% of the BA are cleared from the hepatic circulation for reuse in the liver and 5% are excreted in feces, whereas a small fraction reaches the systemic circulation [7,8]. These circulating BA exert signaling functions in peripheral tissues and organs involved in glucose and lipid metabolism [9,10]. These metabolic effects are driven through the activation of Takeda-G-protein-receptor-5 (TGR5) and the farnesoid X receptor (FXR) in different tissues such as adipose tissue, skeletal muscle, or pancreas [11]. In fact, results from preclinical studies revealed that the activation of TGR5 by BA leads to an increase in energy expenditure and improves glucose and lipid metabolism (3–5).

During exercise, sustained muscle activity is supported by a fine-tunned and coordinated liver response that helps to mobilize energy stores and recycle metabolites, including BA [12]. Several studies have evaluated the effects of EE [13–15] and RE [13] on circulating levels of BA suggesting that the type of exercise might have a different impact on BA levels. In the light of these results, we hypothesize that EE and RE might elicit a differential response on plasma levels of BA. Furthermore, none of the aforementioned studies were conducted in young, sedentary adults or investigated whether the cardiorespiratory fitness (CRF) and muscle strength levels of the individuals, which are well-recognized markers of health status [16,17], could play a role in the circulating levels of BA in response to exercise. Therefore, it is of clinical interest to understand whether it exists an

influence of the individuals' physical fitness status on the circulating levels of BA in response to different types of exercise in humans. Lastly, whether the gut microbiota composition could impact plasma the levels of BA after exercise remains unexplored.

The aim of the present study was to investigate the effect of EE and RE on plasma levels of BA in young, sedentary adults. As a secondary aim, we investigated whether these effects were related to the individual's CRF and muscle strength levels.

METHODS

Individuals and study design

The present study has been conducted under the framework of the ACTIBATE study (ACTivating Brown Adipose Tissue through Exercise; ClinicalTrials.gov ID: NCT02365129) [18]. A total of 14 individuals underwent the EE trial, whereas 17 individuals underwent the RE trials. Inclusion criteria were: i) to be sedentary (i.e., <20min/day of moderate-to-vigorous physical activity in <3days/week); ii) to be non-smoker; iii) not to be taking any medication; and iv) to have a stable body weight over the last 3 months. Exclusion criteria were: having been diagnosed with diabetes, hypertension, or other medical conditions that could be life-threatening or that can interfere with/be aggravated by exercise; being pregnant; being using medication that could affect energy metabolism; and having frequent exposures to cold temperatures.

The study was approved by the Ethics Committee on Human Research of the University of Granada (no. 924) and by the Servicio Andaluz de Salud (Centro de Granada, CEI-Granada) and all individuals signed an informed consent. The study protocol and experimental design were applied following the last revised ethical guidelines of the Declaration of Helsinki.

Acute exercise trials

Both EE and RE trials were performed in a fasted state (i.e., 3-5h fasting), after avoiding stimulants (i.e., caffeine), and avoiding any moderate- and vigorous-intensity exercise (24h and 48h respectively) in separate days.

The EE consisted of a maximum effort test on treadmill (Pulsar treadmill, H/P/Cosmos Sports & Medical GmbH, Nussdorf-Traunstein, Germany) following the modified Balke protocol [19]. Briefly, individuals walked at 3 km/h for 1 min and at 4 km/h for 2 mins for warming up (0% grade) [19]. Then, the test started by walking at 5.3 km/h and 0%. From that moment on, the treadmill

grade was increased by 1% every min, until volitional exhaustion was reached [19]. At this point, individuals started a 5-min recovery walking at 4 km/h and 0% grade [19]. During the whole trial, individuals were equipped with a heart rate monitor (Polar RS800CX, Polar Electro Öy, Kempele, Finland), 10 electrodes for electrocardiogram monitoring, and a Hans-Rudolph plastic mask (model 7400, Hans Rudolph Inc., Kansas City, MO, USA) connected to a preVent™ metabolic flow sensor (Medical graphics Corp, St Paul, MN, USA) for respiratory gas exchange analyses using a CPX Ultima CardioO2 gas exchange analysis system (Medical Graphics Corp, St Paul, MN, USA). During the test, respiratory gas exchange (oxygen consumption (VO_2) and carbon dioxide production) was recorded and the VO_2peak was determined as the highest observed VO_2 value, after excluding artifacts if needed.

The RE consisted on a combination of four different strength tests: i) a maximum isometric strength test in leg press, ii) a handgrip strength test and iii) two 1 repetition maximum (1-RM) tests in bench and leg press [20]. Individuals first completed the maximum isometric strength test in leg press. After being allocated in the leg press machine (A300 Leg Press, Model 2531, Keiser Corporation, Fresno CA, USA), individuals performed two 3-second repetitions, 2 mins apart, for which they were instructed and encouraged to push as hard as they could for the whole duration of the repetition. Afterwards, individuals performed the handgrip strength test by completing two repetitions with each hand, 1 min apart, using a Takei 5401 digital Grip-D hand dynamometer (Takei, Tokyo, Japan) [21]. For the handgrip strength test, individuals remained in a standing position, with the exercising arm parallel and slightly separated from the trunk. The individuals were asked to squeeze the grip gradually and continuously, and as hard as possible. Men executed the test with the grip span of the dynamometer fixed at 5.5 cm, while it was adjusted to the individual's hand size for women, according to a validated equation [21]. The highest strength recorded in each hand was selected and the average between both hands was used for the analyses.

Then, individuals performed the leg press 1-RM test in the above-mentioned leg press machine. After performing 1 set of 10 repetitions with a self-selected light weight for warming-up, they were instructed to perform 1 set of 8 repetitions selecting the resistance with which they could perform 15 repetitions as much. Later, after a 1-min recovery, the resistance load was increased by the study personnel, aiming to set a load with which the individual could perform <10 repetitions, and individuals were instructed to do as many repetitions as possible. The individuals were instructed to stop exercising after 3-4 repetitions if they felt they could perform more than 10 repetitions with the resistance load. If they performed more than 10 repetitions, they rested for 5 mins and repeated

the test with a higher load. The maximum number of attempts for assessing the RM (in a set of <10 repetitions) was 3. Lastly, individuals performed the bench press 1-RM test following the procedure described for the leg press, in a bench within a pneumatic power rack (Power rack, Model 3111, Keiser Corporation, Fresno CA, USA). The 1-RM of both exercises was estimated by the equation previously proposed [22].

Anthropometric and body composition measurements

Weight and height were measured barefoot and wearing light clothing, using a SECA scale and stadiometer (model 799; Electronic Column Scale, Hamburg, Germany). Body mass index (BMI) was calculated from weight and height (kg/m^2). Waist circumference (WC) was measured at the minimum perimeter, at the end of a normal expiration, with the arms relaxed on both sides of the body. WC was measured twice with a plastic tape measure; the two measures were averaged for further analyses. Lean, fat, and visceral adipose tissue (VAT) masses were measured by dual-energy X-ray absorptiometry using a Discovery Wi device (Hologic Inc., Bedford, MA, USA) equipped with analysis software (APEX version 4.0.2). Fat mass was also expressed as a percentage of body weight and the lean and fat mass indices as kg/m^2.

Blood sample collection and determination of cardiometabolic risk factors

Fasting blood samples were drawn from the antecubital vein in the morning (8.00–9.00 A.M) after overnight fasting (>10 h). Blood samples were collected in Vacutainer Tubes® and centrifuged, obtaining serum (obtained with Vacutainer® SST™ II Advance tubes) and plasma (obtained with Vacutainer® Hemogard™ tubes, containing potassium salt of ethylenediamine tetra-acetic as anticoagulant) aliquots that were stored at -80°C until analyses. For the acute exercise trials the blood was collected before, and 3, 30, 60 and 120 min after the end of each exercise session, and plasma aliquots were stored at -80°C until lipidomics analyses.

Glucose levels were assessed using an AU5832 analyser (Beckman Coulter, Brea, CA, USA) with a Beckman Coulter reagent (OSR6521). Insulin was measured by chemiluminescence immunoassays using the UniCel DxI 800 analyser (Beckman Coulter) with Beckman Coulter chemiluminescent reagent (33410). TC, HDL-C, TG, glutamic pyruvic transaminase (GTP), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), and creatinine were measured using an AU5832 spectrophotometer (Beckman Coulter) with Beckman Coulter reagents (OSR6116, OSR60118, OSR6187, OSR6507, OSR6520, OSR6204, and OSR6678). LDL-C (mM) was calculated from the Friedewald formula $[TC\ (mM) - HDLc(mM) - 0 \cdot 45 \times TG\ (mM)$. C-reactive protein, C3 protein, and C4 protein were measured by immunoturbidimetric assays (OSR6299, OSR6159,

and OSR6160) using an AU5832 spectrophotometer. Leptin and adiponectin were measured in plasma using the MILLIPLEX MAG Human Adipokine Magnetic Bead Panel 2 (Catalogue # HADK2MAG-61K) and a MILLIPLEX MAP Human Adipokine Magnetic Bead Panel 1 (Catalogue # HADK1MAG-61K), respectively (Luminex Corporation, Austin, TX, USA). Systolic and diastolic blood pressure was measured at three different consecutive time points using an automatic Omrom M2 (Omron Healthcare, Kyoto, Japan) following the guidelines of the European Heart Society [23]. The homeostasis model assessment (HOMA index), and fatty liver index (FLI) were then calculated [24].

Determination of plasma bile acids

Plasma levels of BA were determined with a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) method [25]. Briefly, plasma samples were prepared with a liquid-liquid extraction method and analyzed using a Shimadzu LC system (Shimadzu Corporation, Kyoto, Japan) connected to a SCIEX QTRAP 6500+ mass spectrometer (AB Sciex, Framingham, MA, USA). The protocol enabled the relative quantitation of primary (i.e., CA, CDCA, glycocholic acid [GCA], and glycochenodeoxycholic acid [GCDCA]) and secondary (i.e., DCA, glycodeoxycholic [GDCA], glycolithocholic [GLCA], glycoursodeoxycholic [GUDCA]) BA. The area peak ratio of primary and secondary BA measured summed from the individual data, and the new computed variables were expressed as total BA, primary BA, and secondary BA. The BA detected by this method are listed in **Table 1,** whereas the internal standards used are in **Table 2**.

Table 1. List of bile acids analyzed, including relative standard deviations (RSDs) observed in the quality control (QC) samples.

Abbreviation	Name (International Union of Pure and Applied Chemistry, IUPAC)	ChEBI ID	RSD in QC
Primary bile acids			
CA	3α,7α,12α-trihydroxy-5β-cholan-24-oic acid	16359	4.4%
GCA	N-(3α,7α,12α-trihydroxy-5β-cholan-24-oyl)-glycine	17687	3.6%
CDCA	3α,7α-Dihydroxy-5β-cholan-24-oic Acid	16755	7.0%
GCDCA	N-(3α,7α-dihydroxy-5β-cholan-24-oyl)-glycine	12544	5.3%
Secondary bile acids			
DCA	3α,12α-Dihydroxy-5β-cholan-24-oic Acid	28834	6.2%
GDCA	N-(3α,12α-dihydroxy-5β-cholan-24-oyl)-glycine	27471	5.3%
GLCA	N-[(3α,5β)-3-hydroxy-24-oxocholan-24-yl]-glycine	37998	8.4%
GUDCA	N-(3α,7β-dihydroxy-5β-cholan-24-oyl)-glycine	89929	4.3%

Observed variability is expressed as relative standard deviation of the peak area ratio in the quality control samples. *Abbreviations*: CA: cholic acid; CDCA: chenodeoxycholic acid; ChEBI: Chemical Entities of Biological Interest; DCA: deoxycholic acid; GCA: glycocholic acid; GCDCA: glycochenodeoxycholic acid; GDCA: glycodeoxycholic acid; GLCA: glycolithocholic acid; GUDCA: glycoursodeoxycholic acid; QC: quality control; RSD: relative standard deviation.

Table 2. List of internal standards used in the LC-MS/MS method.

Abbreviation	Name (International Union of Pure and Applied Chemistry, IUPAC)
d4-CA-ISTD	3α,7α,12α-trihydroxy-5β-cholan-24-oic acid-d4
d4-GCA-ISTD	N-(3α,7α,12α-trihydroxy-5β-cholan-24-oyl)-glycine-d4
d4-DCA-ISTD	3α,12α-dihydroxy-5β-cholan-24-oic acid-d4
d4-GDCA-ISTD	N-(3α,12α-dihydroxy-5β-cholan-24-oyl) glycine-d4
d5-GUDCA-ISTD	N-(3α,7β-dihydroxy-5β-cholan-24-oyl)-glycine-d5

Abbreviations: CA: cholic acid; DCA: deoxycholic acid; GCA: glycocholic acid; GDCA: glycodeoxycholic acid; GUDCA: glycoursodeoxycholic acid.

Sample preparation

The sample preparation was performed on ice, except for the evaporation step. BA were extracted using liquid-liquid extraction [25]. Before the extraction, 150 µL of plasma sample was transferred into a 1.5 mL Eppendorf tube and mixed with 5 µL of an antioxidant solution composed of 0.4 mg/mL of butylated hydroxytoluene (BHT), and 10 µL of an internal standard solution containing the isotopically labeled analogs (**Table S2**). Then, 150 µL of buffer solution (0.2 M citric acid and 0.1 M disodium hydrogen phosphate at pH 4.5) were added, followed by the addition of 1 mL extraction solvent composed of methyl-tertbutyl-ether and butanol (50:50, *v*/*v*). Samples were then mixed for 5 min using a bullet blender (Next Advance, Averill Park, NY) and centrifugated (16,000 g, 10 min, 4 °C). Next, 900 µL of supernatant were transferred to a new 1.5 mL Eppendorf tube and evaporated to dryness using a SpeedVac system at room temperature. The dry residue was reconstituted in 50 µL of methanol:acetonitrile (70:30, *v/v*), and centrifuged (16,000 g, 10 min, 4 °C). Finally, 40 µL of the supernatant was transferred into an autosampler vial and 10 µL was injected into the LC-MS/MS system.

Liquid chromatography-tandem mass spectrometry

LC-MS/MS analysis was performed as previously described [25]. Briefly, the extracted samples were analyzed using a Shimadzu LC system (Shimadzu Corporation, Kyoto, Japan), coupled to a SCIEX QTRAP 6500+ mass spectrometer (SCIEX, Framingham, MA). The separation was carried out using a BEH C18 column (50 mm × 2.1 mm, 1.7 µm) from Waters Technologies (Milford, MA) kept at 40 °C. The mobile phase consisted of 0.1% acetic acid in water (A), 0.1% acetic acid in acetonitrile/methanol (90:10, *v/v*, B) and 0.1% acetic acid in isopropanol (C). The data acquisition was performed using electrospray ionization in negative mode. MS/MS acquisition was carried out using Selected Reaction Mode (SRM). SRM transitions were individually optimized for targeted analytes and their respective internal standards using standard solutions.

Data pre-processing

For each target compound, the ratio between its peak area and the peak area of its respective internal standard was calculated using SCIEX OS-MQ Software and was used for further data analysis. The data quality was monitored using regular injection of quality control (QC) samples, prepared from of blank plasma samples. QC samples were used to correct for between batch variations, using the in-house developed mzQuality workflow (available at http://www.mzQuality.nl) [25]. Relative standard deviations (RSDs) were calculated for each analyte present in the quality control (QC) samples (**Table 1**). All analytes showed RSD values in QC samples below 10%, ensuring high data quality.

Assessment of ^{18}F-fluorodeoxyglucose uptake by brown adipose tissue

Briefly, the shivering threshold of each individual was determined following a personalized cooling protocol [26] 48 to 72 hours after the shivering threshold determination, individuals were exposed to a 2-hour personalized cooling procedure at 3·8ºC above their individual shivering threshold. After 1 hour of cold exposure, a bolus of ~185 MBq of ^{18}F-fluorodeoxyglucose (^{18}F-FDG) was intravenously injected and the water temperature was increased by 1ºC. If subjects reported shivering, the water temperature was further increased by 1ºC. After 2 hours of cold exposure, a static positron emission tomography/computed tomography (PET/CT, Siemens Biograph 16 PET-CT, Erlangen, Germany) scan was performed. CT acquisition was performed using a peak of 120 kV and PET acquisition with a scan time of 6 minutes per bed position. PET/CT images were obtained from the atlas (i.e., cervical vertebra 1) to approximately the mid-chest. BAT-related outcomes were calculated as described [27].

Fecal microbiota analysis

A fecal sample (50-60 g) was obtained from each volunteer and introduced in a 60 mL plastic sterile container. The fecal samples were transported in a portable cooler with an ice plate to the research center and stored at -80ºC until DNA extraction. Fecal samples were homogenized in a Stomacher® 400 blender (A. J. Seward and Co. Ltd., London, UK). The DNA extraction and purification steps were performed with a QIAamp DNA Stool Mini Kit (QIAGEN, Barcelona, Spain) according to the manufacturer's instructions. DNA concentration and purity were determined with a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, DE, USA).

Sequencing

Extracted DNA was amplified by PCR targeting the V3 and V4 hypervariable regions of the bacterial 16S rRNA gene using the following primer pairs: 16S Amplicon PCR Forward Primer: 5´CCTACGGGNGGCWGCAG; and 16S Amplicon PCR Reverse Primer: 5'GACTACHVGGGTATCTAATCC. The PCR assays were carried out in a 25 µL final reaction volume, including 12.5 µL of the 2X KAPA HiFi Hotstart prepared mixture (KAPA Biosystems, Woburn, MA, USA), 5 µL of each forward and reverse primer (1 µM), and 2.5 µL of extracted DNA (10 ng). The following PCR program was used: (i) denaturation at 95 °C for 3 min, (ii) 8 denaturation cycles at 95 °C for 30 s, (iii) annealing at 55 °C for 30 s, (iv) elongation at 72 °C for 30 s, (v) final extension at 72 °C for 5 min. Next, AMPure XP microspheres (Beckman Coulter, Indianapolis, IN, USA) were used to purify the 16S V3 and V4 amplicon away from free primers and primer-dimer species. For the PCR index step, we used the Nextera XT index kit (Illumina, San Diego, CA, USA) to tag DNA with the sequencing adapters. The pooled PCR products were purified using AMPure XP balls (Beckman Coulter, Indianapolis, IN, USA) before quantification. The amplicons were sequenced in a MiSeq (Illumina, San Diego, CA, USA), using the Illumina MiSeq paired-end sequencing system (2x300nt) (Illumina, San Diego, CA, USA).

Fecal microbiota bioinformatics

The "*Dada2*" [28] package version 1.10.1 in *R* software [29] was used for analyzing the raw sequences (FastQ files). All samples that were above the 10,000 reads cut-off threshold were considered valid for subsequent analyses. Samples were standardized to an equal sequencing depth of 30,982 reads using the "*Phyloseq*" [30] package in *R* software, leading to a total of 11,158 different phylotypes.

Phylotypes were assigned to their specific taxonomic affiliation (from phylum to genus) based on the naïve Bayesian classification with a pseudo-bootstrap threshold of 80% [30](using the "*Classifier*" function in Ribosomal Data Project (RDP) [31]. We obtained a total of 209 genera that belong to 16 different phyla. In order to determine the species taxonomies, we used the "*Seqmatch*" function in RDP. For the main analysis, we used relative abundances as the read's percent of each phylotype relative to the total number of reads. We performed the analyses when the average of the relative sequence abundance was higher than 1%. Only those species with ≥ 97% coincidence with the respective representative sequence read and found in at least 50% of the individuals were annotated, identifying a total of 50 different species for the analyses.

Beta diversity indicates the number of species shared among the microbial community from the individuals, and was assessed by pseudo-F statistics. Pseudo-F shows the ratio between cluster variance and within-cluster variance [(between-cluster-sum-of-squares/(c-1))/(within-cluster-sum-of-squares/(n-c))], where c is the number of clusters and n is the number of variables [32]. A pseudo-F value of 1 indicates that the variance between and within-group is similar; if pseudo-F ≥ 1, the between-group variance is higher than the within-group variance. Alpha diversity reflects the number of different phylotypes and the relative abundance of these phylotypes within the same individual. A total of 4 different alpha diversity indexes were calculated: i) *species richness* (number of different phylotypes in the community) ii) *evenness index* (equitability of the phylotypes frequencies in the community) [33]; iii) *Shannon index* (number and equitability of the phylotypes in the community) [34]; iv) *inverse Simpson index* (derived from the classical Simpson index; richness in a community with uniform evenness) [35].

Statistical analyses

Descriptive data are expressed as mean ± standard deviation, unless otherwise stated. First, data normality was checked using the Shapiro-Wilk test, visual histograms, and Q-Q plots. None of the BA followed a normal distribution, thereby all values were log2-transformed for the analyses. The effects of EE and RE on plasma levels of BA were analysed by one-way repeated-measures analysis of variance (ANOVA). In this test, the different time when blood was collected (i.e., baseline, 3, 30, 60, and 120 min) was included as 'time'. These fold changes relative to baseline values were calculated with the log2-transformed outcomes (e.g., 120 min fold change = log2 area peak ratio at 120 min minus log2 area peak ratio at baseline). Next, the function "Visual Binning" of SPSS (Statistical Package for the Social Sciences v.26.0; IBM Corporation, Chicago, IL, USA) was used to divide the cohort into low/high CRF individuals and low/high muscle strength individuals based on the median levels of the VO_{2peak} relative to body weight and RM leg press respectively. Then, two-factor-repeated measures ANOVA were performed to investigate if the effects of the of EE and RE were different depending on the CRF and muscle strength levels (low/high CRF levels or low/high muscle strength levels and time). The differences in specific time points between low/high CRF individual were assessed by t-tests for independent samples comparing the fold change of each time point. We performed t-tests for independent samples to investigate whether the differences in CRF levels were related to differences in body composition and metabolic status of the individuals. Additionally, analyses of covariance adjusting for fat mass percentage were performed to study if differences in the metabolic status

were independent of the individual's adiposity. All analyses were performed using the Statistical Package for the Social Sciences v.26.0 (IBM Corporation, Chicago, IL, USA) and figures were built with GraphPad Prism software v.9 (GraphPad Software, San Diego, CA, USA). The statistical significance was set at $P<0.05$.

RESULTS

The characteristics of the individuals involved in both EE and RE interventions are shown in **Table 3.**

Table 3. Baseline characteristics of the study individuals.

	Endurance (n=14)		**Resistance (n=17)**	
	Mean	SD	Mean	SD
Demographics				
Age (years old)	21.8	2.5	22.4	2.5
Male (%)	2	14%	6	35%
Female (%)	12	86%	11	64%
Body composition				
BMI (kg/m^2)	24.2	4.0	25.3	4.2
Lean mass (kg)	39.6	7.2	41.8	9.0
Fat mass (kg)	24.1	9.5	26.2	6.7
Fat mass (%)	35.9	10.0	37.1	6.3
VAT mass (g)	326	173	378	160
Physical Fitness				
RM leg press (kg)	205.2	54.8	210.3	69.6
VO_2peak (mL/kg/min)	40.7	7.2	40.0	9.6
Time to exhaustion (s)	806	236	872	219

Data are presented as mean and standard deviation (SD), otherwise stated. *Abbreviations*: BMI: body mass index; RM: repetition maximum; VAT: visceral adipose tissue; VO_2: oxygen consumption.

Plasma levels of bile acids transiently decrease after a bout of endurance and resistance exercise in a different manner

EE rapidly decreased plasma levels of total and secondary BA until 30 min after exercise (-52% and - 45%, respectively), whereas primary BA decreased just after 3 min after exercise (-59%) (**Fig. 1A**; all $P\leq0.014$, $\eta^2\geq0.272$). From there on, plasma levels of total and secondary BA tend to increase their levels until 120 min, while primary BA returned nearly to their baseline levels at 120 min (-18%). The analysis of the kinetics of each BA revealed that EE lowered plasma levels of the conjugated primary BA GCA and GCDCA 30 min after exercise (-79% and -28%,

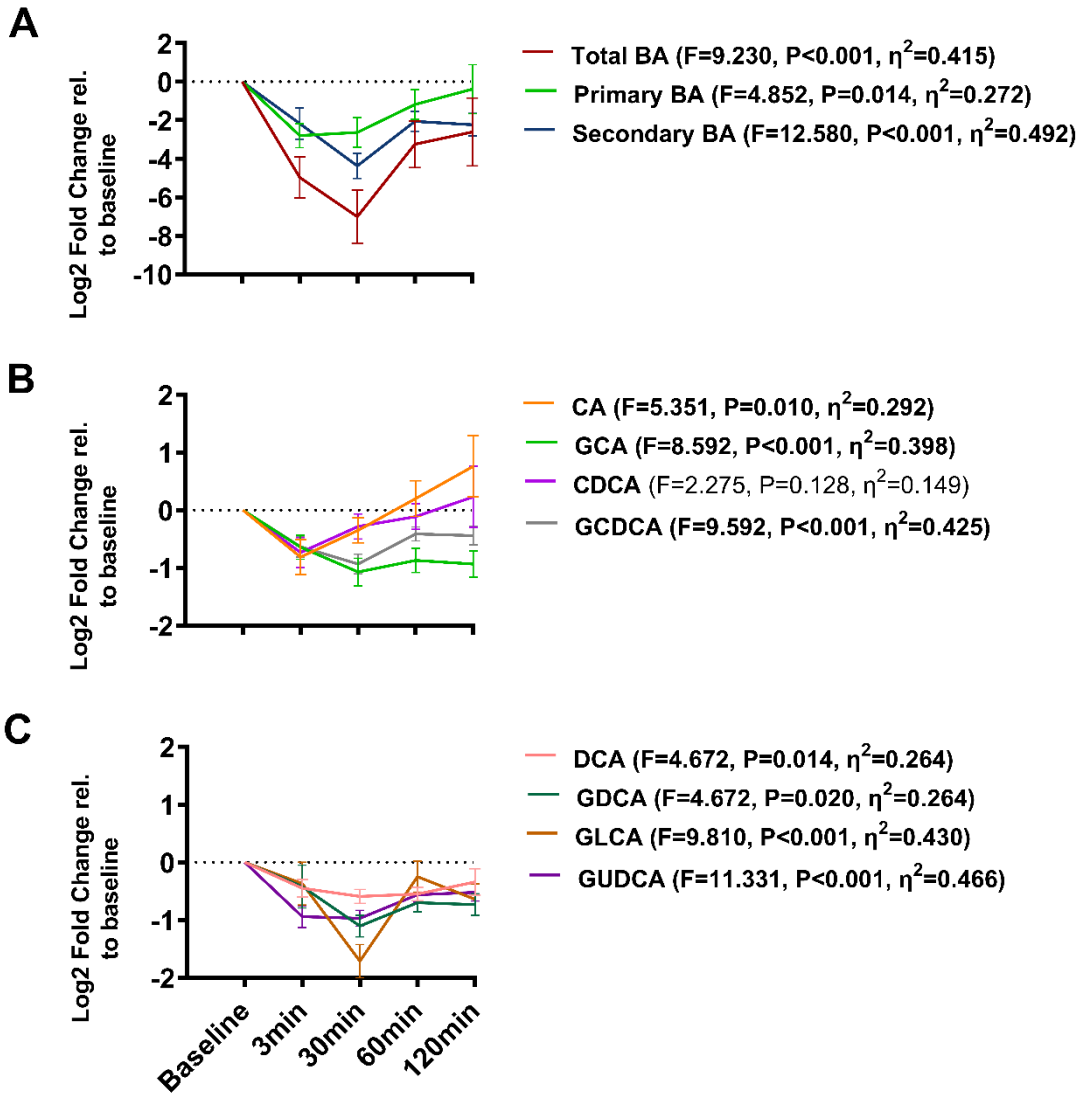

Figure 1. Acute endurance exercise rapidly and transiently reduces the plasma levels of total, primary and secondary bile acids (n=14). Changes in bile acid groups (A), primary (B) and secondary (C) bile acids after acute endurance exercise. Each line represents the kinetics of the mean log2 fold change relative to baseline of each group of bile acids or individual bile acids. The sum of total, primary and secondary bile acids were calculated. F, P and η^2 values obtained from repeated measures analyses of variance (ANOVA). *Abbreviations*: CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; GCA: glycocholic acid; GCDC: glycochenodeoxycholic acid; GDCA: glycodeoxycholic acid; GLCA: glycolithocholic acid; GUDCA: glycoursodeoxycholic acid.

respectively), while the unconjugated primary BA CA decreased until 3 min (-42%; **Fig. 1B**; all P ≤0.010, η^2≥0.292). From that point, plasma levels of GCA and GCDCA remained moderately stable, while CA continued increasing its concentration until surpassing its baseline levels 120 min after exercise (+40%; **Fig. 1B**; all P ≤0.010, η^2≥0.292). The secondary BA DCA, GDCA, GLCA and GUDCA decreased their concentration until 30 min after EE (-20%, -94%, -61%, and -34%, respectively), but their levels remained relatively steady from there on (**Fig. 1C;** all P≤0.020, η^2≥0.264). On the other hand, RE exclusively elicited a rapid reduction of plasma levels of secondary BA, reaching their lowest concentration

at 120 min post exercise (-26%; **Fig. 2A**; P<0.001, η^2=0.270). Specifically, RE reduced the concentration of the conjugated primary BA GCA and GCDCA until min 120 (-68% and -17%, respectively) (**Fig. 2B**; all P≤0.005, η^2≥0.205) and the plasma levels of the conjugated secondary BA GDCA, GLCA, and GUDCA until 120 min after RE (-101%, -39%, and -17%; **Fig. 2C**; all P≤0.020, η^2≥0.187).

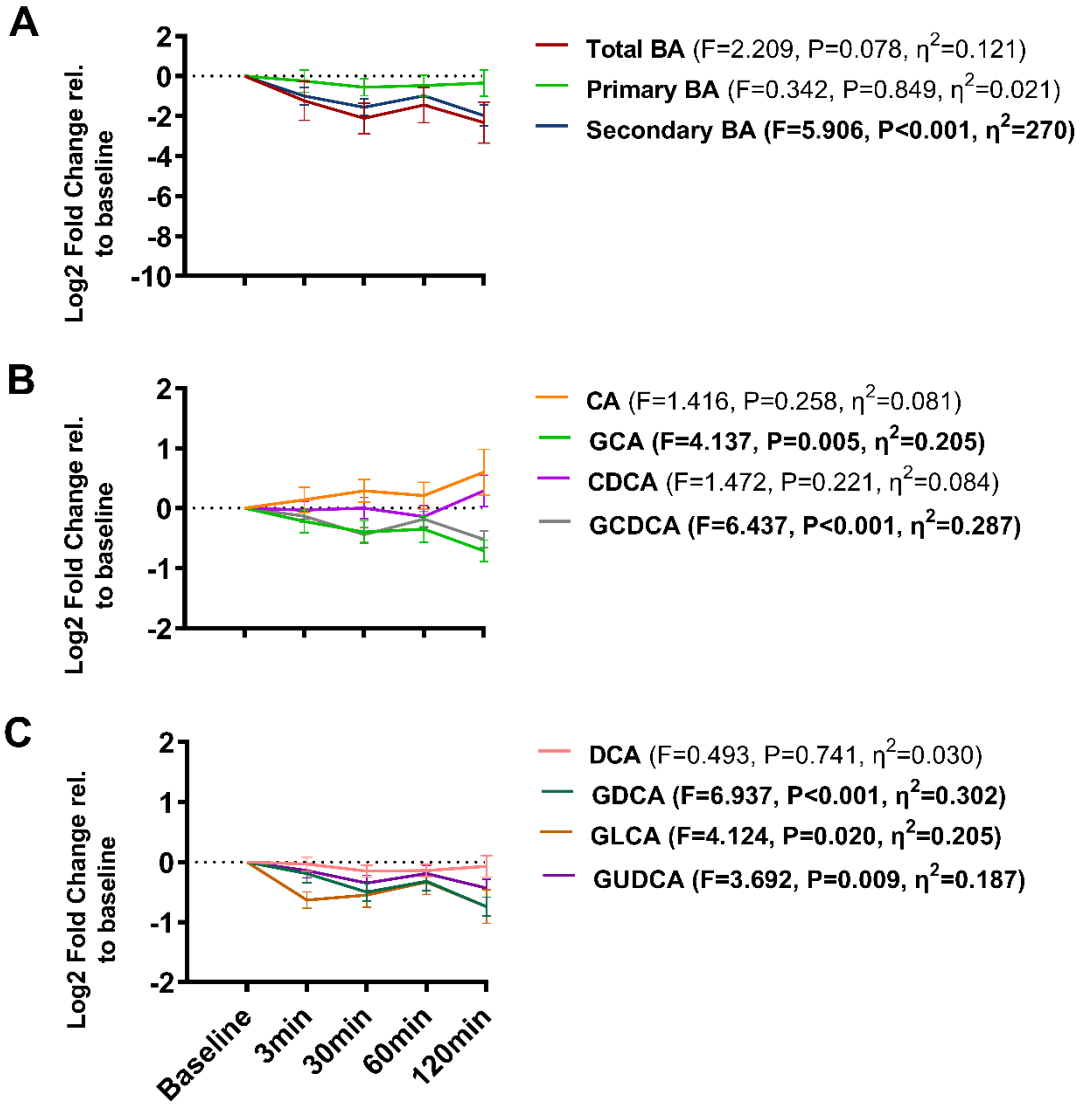

Figure 2. Acute resistance exercise exerts a rapid and prolonged reduction of plasma levels of secondary bile acids (n=17). Changes in bile acid groups (A), primary (B) and secondary (C) bile acids after acute resistance exercise. Each line represents the trajectory of the mean log2 fold change relative to baseline of each group of bile acids or individual bile acids. The sum of total, primary and secondary bile acids were calculated. F, P and η^2 values obtained from repeated measures analyses of variance (ANOVA). *Abbreviations*: CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; GCA: glycocholic acid; GCDC: glycochenodeoxycholic acid; GDCA: glycodeoxycholic acid; GLCA: glycolithocholic acid; GUDCA: glycoursodeoxycholic acid.

Individuals with high cardiorespiratory fitness levels show a significantly higher increase in plasma levels of primary unconjugated primary bile acids levels after 120 mins of acute endurance exercise compared to their low cardiorespiratory fitness levels counterparts

To gain insight into whether the individual's CRF and strength levels play a role in the kinetics of plasma BA in response to exercise, the EE cohort was divided into individuals with low and high CRF levels. We found that the kinetics of plasma levels of primary BA after EE were different across individuals with high and low CRF levels (**Fig. 3B**; $P_{interaction}$=0.044).

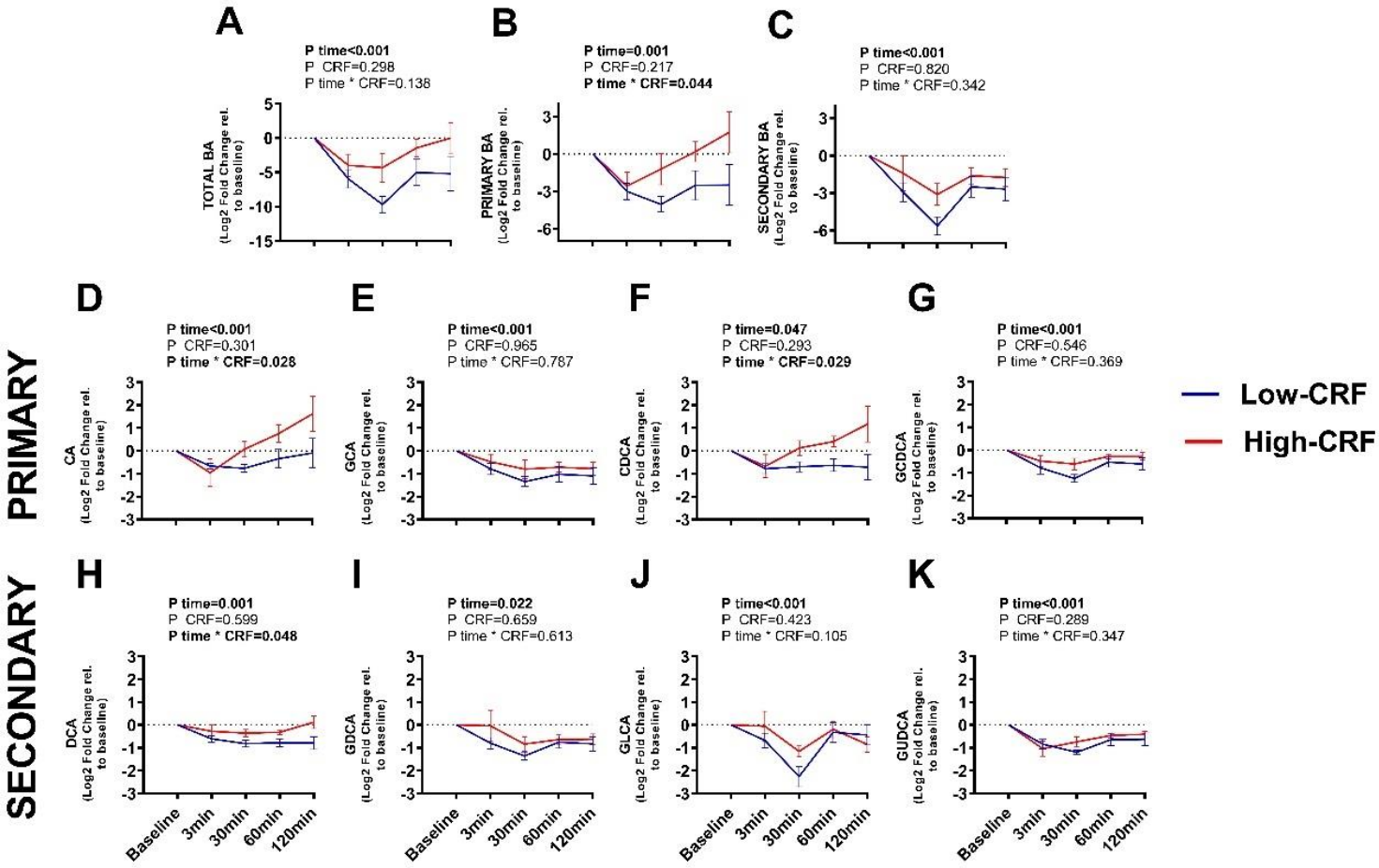

Figure 3. The response in plasma levels of bile acids after acute endurance exercise is different between young adults with low and high cardiorespiratory fitness levels. Each line represents the kinetics of the mean log2 fold change relative to baseline of each group of bile acids or individual bile acids. The sum of total (A), primary (B) and secondary (C) bile acids were calculated. P values obtained from two-factor (low/high CRF and time) repeated measures analyses of variance (ANOVA). *Abbreviations*: CA: cholic acid; CDCA: chenodeoxycholic acid; CRF: cardiorespiratory fitness; DCA: deoxycholic acid; GCA: glycocholic acid; GCDC: glycochenodeoxycholic acid; GDCA: glycodeoxycholic acid; GLCA: glycolithocholic acid; GUDCA: glycoursodeoxycholic acid.

Thus, while primary BA acutely decreased in both low and high CRF levels groups 3 min after exercise (-64% and -65%, respectively), individuals with high CRF levels showed an increase in plasma levels of primary BA from there on until they exceeded their baseline levels at 120 min (+37%; **Fig. 3B**) whereas in their counterparts primary BA remained under their baseline levels at 120 min (-53%; **Fig. 3B**). Particularly, there were significant differences in the kinetics of plasma levels of the unconjugated primary BA CA (**Fig. 3D**; $P_{interaction}$=0.028) and CDCA (**Fig. 3F**; $P_{interaction}$=0.029), which displayed a unique trend to increase 120 min after finishing the EE above their baseline levels exclusively in the high CRF group (high CRF= +77% vs low CRF= -5%, **Fig. 3D**; and high CRF= +65% vs Low= CRF -39%, **Fig. 3F**, respectively).

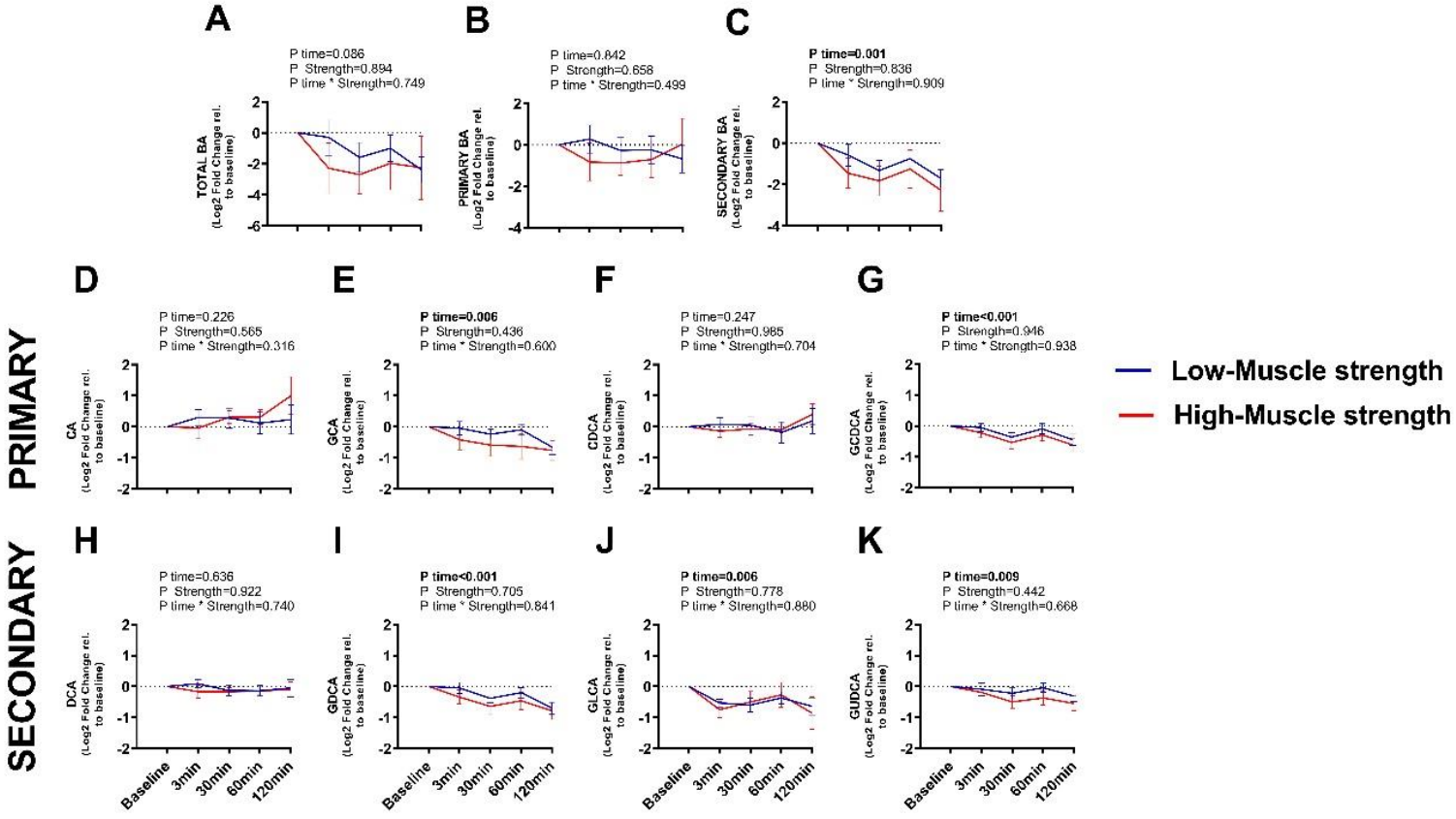

Figure 4. The response in plasma levels of bile acids after acute resistance exercise is similar between young adults with low and high lower body strength levels. Each line represents the trajectory of the mean log2 fold change relative to baseline of each group of bile acids or individual bile acids. The sum of total (A), primary (B) and secondary (C) bile acids were calculated. P values obtained from two-factor (low/high CRF and time) repeated measures analyses of variance (ANOVA). *Abbreviations*: CA: cholic acid; CDCA: chenodeoxycholic acid; CRF: cardiorespiratory fitness; DCA: deoxycholic acid; GCA: glycocholic acid; GCDC: glycochenodeoxycholic acid; GDCA: glycodeoxycholic acid; GLCA: glycolithocholic acid; GUDCA: glycoursodeoxycholic acid.

Likewise, the RE cohort was divided into individuals with low and high lower and upper strength levels. Contrary to EE, there were no differences in the kinetics of plasma levels of BA after RE between individuals with low and high levels of low muscle strength (leg press) (**Fig. 4**; all $P_{int} \geq 0.316$), neither between individuals with low and high levels of low muscle strength (bench press; $P_{int} \geq 0.05$, data not shown) or with low and high handgrip strength levels ($P_{int} \geq 0.05$, data not shown).

Individuals with high cardiorespiratory fitness levels present higher adiposity levels and a worse cardiometabolic profile than their low cardiorespiratory fitness levels counterparts, but similar fecal microbiota composition

We then investigated whether the aforementioned differences in BA kinetic after EE could be related to the body composition and metabolic status of the individuals. Interestingly, individuals with high CRF levels presented lower BMI (-21%) and adiposity levels (-34% fat mass percentage; -47% VAT mass), and a better glucose (-45% insulin levels; -49% HOMA index) and immuno-metabolic profile (-77% serum leptin; -58% C-reactive protein; -15% creatinine, and -16% C3 levels) in comparison to their low CRF levels counterparts (**Table 4**; all P≤0.045). However, no differences were observed for hepatic enzymes, FLI, blood pressure, lipid, and BAT parameters (**Table 4**; all P>0.05). The differences in glucose and immuno-metabolic parameters persisted after adjusting for fat mass % (**Table 4**; all P≤0.045).

Finally, due to the unequivocally role of gut microbiota on BA metabolism, we compared the fecal microbiota composition between individuals with high and low CRF levels. The results revealed no differences in beta or alpha diversity (**Fig. 5A-B**; all P≥0.295) or in relative abundance at phylum and genus levels (**Fig. 5C**; all P>0.05).

Table 4. Baseline characteristics of the individuals that performed acute aerobic exercise (n=14) after dividing by the levels of cardiorespiratory fitness.

		Low CRF (n=7)		**High CRF (n=7)**		**P**	**P_1**
		Mean	SD	Mean	SD		
Sex (n, con %)						0.127	-
	Men	0	0	2	29		
	Women	7	100	5	71		
Age (years)		22.84	2.37	20.7	2.3	0.112	0.167

Body composition						
BMI (kg/m²)	27.0	3.7	21.3	1.6	**0.006**	**0.012**
WC (cm)	82.0	9.7	76.0	8.7	0.24	0.498
Lean mass (kg)	39.7	5.2	39.5	9.2	0.969	**0.009**
LMI (kg/m²)	14.3	1.6	14.2	2.3	0.94	0.074
Fat mass (kg)	31.9	5.6	16.2	4.7	**<0.001**	-
FMI (kg/m²)	11.5	2.2	6.0	2.1	**<0.001**	-
Fat mass (%)	43.2	3.1	28.7	9.1	**0.002**	-
VAT (g)	426.2	178.5	224.9	95.1	**0.022**	-
Brown adipose tissue						
BAT volume (mL)	83.1	57.1	79.6	41.3	0.903	0.214
BAT SUVmean	4.0	3.1	4.6	1.7	0.711	0.466
BAT SUVpeak	16.0	14.9	15.4	6.9	0.932	0.669
BAT Radiodensity	-58.9	12.8	-50.5	5.8	0.27	0.659
Cardiometabolic risk factors						
Glucose (mg/dL)	89.9	4.5	85.1	6.2	0.125	0.316
Insulin (µIU/mL)	10.9	5.5	6.0	1.1	**0.015**	**0.045**
Insulin glucose ratio	17.9	7.6	11.0	2.8	**0.033**	0.08
HOMA index	2.5	1.4	1.3	0.2	**0.012**	**0.039**
Creatinine (mg/dL)	0.7	0.1	0.8	0.1	0.05	**0.012**
GTP (IU/L)	18.9	13.9	17.1	11.9	0.774	0.902
GGT (IU/L)	23.9	24.7	14.6	3735.0	0.552	0.669
ALP (IU/L)	63.1	16.5	56.7	16.9	0.447	0.383
Fatty liver index	14.4	10.9	5.8	2.9	0.113	0.124
Total cholesterol (mg/dL)	169.1	48.6	175.0	35.9	0.722	0.264
HDL-C (mg/dL)	49.7	6.6	56.0	10.9	0.214	0.369
LDL-C (mg/dL)	103.7	41.9	106.0	31.2	0.79	0.293
Triglycerides	78.4	44.1	65.1	18.8	0.591	0.654
C-reactive protein (mg/L)	2.9	2.1	1.2	1.7	**0.033**	**0.023**
C3 (mg/dL)	152.7	14.3	127.5	22.5	**0.024**	**0.025**
C4 (mg/dL)	33.9	10.2	25.3	9.3	0.086	0.224
Leptin (µg/L)	11.6	4.6	2.7	2.2	**0.006**	**<0.001**
Adiponectin (mg/L)	10.9	6.3	15.7	10.3	0.316	0.524
Systolic blood pressure (mmHg)	116.9	9.1	116.7	15.8	0.977	0.178
Diastolic blood pressure (mmHg)	72.5	4.8	70.1	6.6	0.451	0.502

Data are presented as mean and standard deviation (SD), otherwise stated. *Abbreviations*: ALP: alkaline phosphatase; BAT: brown adipose tissue; BMI: body mass index; C3: complement component 3; C4: complement component 4; FMI: fat mass index; GGT: gamma-glutamyl transferase; GTP: glutamic pyruvic transaminase; HDL-c: high-density lipoprotein cholesterol; HOMA: homeostasis model assessment LDL-C: low-density lipoprotein cholesterol; LMI: lean mass index; RM: repetition maximum; SUV: standardized uptake value; VAT: visceral adipose tissue; WC: waist circumference. P values are derived from Student's t-test for independent samples. P_1 values are derived from the analyses of covariance adjusting for fat mass percentage. Boldfaced values mean $P < 0.05$. For statistical analyses, serum levels of cardiometabolic risk factors were log10 transformed.

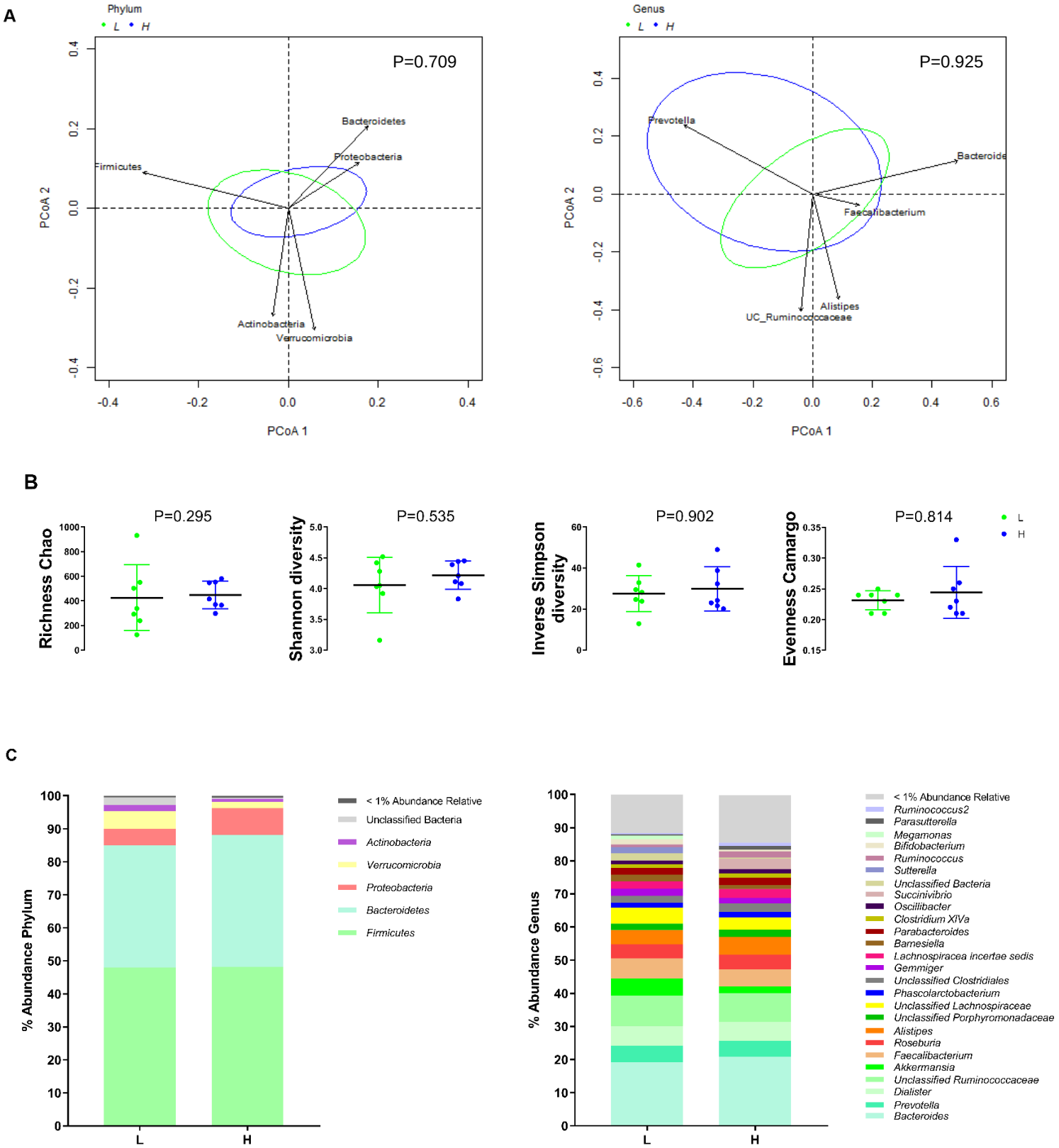

Figure 5. Fecal microbiota diversity and composition is similar between subjects with low and high cardiorespiratory fitness levels. (L: Low, n=7; H: High, n=7). Panel A shows Principal Coordinate Analysis (PCoA) plot of the first two principal coordinates at phylum and genus level, categorized by groups of cardiorespiratory fitness. Genus PCoA only shows the five genera of higher abundance. For this analysis, only microorganism whose abundance relative were higher that 0.5%, were included. PCoA analyses were done using Bray-curtis dissimilarity. Ellipses represent the 95% CI. Panel B shows the differences between the groups of cardiorespiratory fitness in fecal microbiota diversity indexes (richness Chao, Shannon, inverse of Simpson and evenness Camargo). Mann-Whitney test ($p<0.05$) was used to test for each pairwise comparison (GraphPad Prism 8.00). Panel C indicates relative abundance of the fecal microbiota at phylum level and genus level according to cardiorespiratory fitness levels. Stacked bar represented percentage abundance.

DISCUSSION

Here, we investigated the effects of EE and RE on plasma levels of BA in young, sedentary adults. Overall, EE acutely decreased plasma levels of total, primary and secondary BA during the first 30 mins after exercise, but their levels slightly increased there on, especially the primary BA that almost returned to their baseline levels at 120 min after exercise. On the other hand, acute RE exclusively reduced the plasma levels of secondary BA, yet this decrease was persistent and remained stable 120 min after exercise. Notably, the kinetics of plasma levels of primary BA CA and CDCA after EE were different across individuals with low and high CRF levels: after decreasing in a similar fashion in both groups 3 min after exercise, individuals with higher CRF levels showed an increase in plasma levels of primary BA CA and CDCA from there on until surpassing their baseline levels at 120 min (+77% and +65, respectively), in contrast to their counterparts in whom primary BA remained decreased at 120 min after exercise (-5% and -39%, respectively). Contrary, no differences were found in the kinetics of plasma levels of BA after acute RE between individuals with low and high muscular strength levels. Further analyses revealed that those individuals with high CRF levels presented lower adiposity levels and an improved glucose and immuno-metabolic profile in comparison to their counterparts, but similar fecal microbiota composition. This suggest that the increase in CA and CDCA 120 min after EE is linked to their better health status of high CRF individuals, independently of their fecal microbiota composition.

A bout of endurance exercise rapidly and transiently reduces the plasma levels of bile acids

Overall, our findings revealed that acute EE decreased plasma levels of total, primary, and secondary BA during the first 30 min after exercise, followed by a slightly increase that was more pronounced in the case of primary BA. This is in contrast to the results of a previous study where plasma levels of total, primary and secondary BA did not change after 60 min of EE on a cycloergometer at 70% VO_2peak in recreationally active males [13]. Pursuant to our EE results, running 21 km significantly reduced the serum concentration of total BA by (~-46%), specifically CA, DCA and GUDCA, in 30 middle-aged recreational just after finishing the exercise [14]. A third study found a reduction of plasma levels GCA and DCA immediately after finishing a longer running session (80.5 km, treadmill) in trained male runners [15]. However, the type of endurance exercise [13] (i.e., cycling vs walking), duration and intensities of these studies [13–15] significantly differed from our EE protocol. Furthermore, they included active individuals with higher CRF levels [13–15] than our sedentary individuals. This fact precludes us to draw firm conclusions, because CRF clearly influences the

circulating levels of metabolites in response to EE [3]. Despite these differences, the results across these studies seem shows that an extenuating EE, either the case of our study (maximum walking effort test), running 21km [14], or 80.5 km [15], acutely reduces the circulating levels of BA in the first 30 min after exercise in humans.

A bout of resistance exercise exert a prolonged reduction of plasma levels of bile acids

In regards to RE, the same 10 individuals of the study of Morville et al. performed a RE session on a separate day [13]. The RE protocol consisted of 5 resistance drills, conducting 5 sets of 10 repetitions with 90 secs of rest between sets, with a total session duration of 60 min. Contrary to our findings, they reported that RE reduced total plasma levels of BA 60 and 180 min after finishing the exercise (-35% and -41%, respectively). Specifically, RE reduced plasma levels of CA (-69% at 60 and 180 min), CDCA (-55% at 120min, -52% at 180 min), and GUDCA (-58% immediately after, and -83% at min 60 and 180) [13]. The differences between RE protocols might explain the discrepancies with our results since they conducted a more extenuating RE protocol in terms of intensity and duration. Overall, the results of Morville et al. suggest that the plasma levels response of BA to acute exercise might be exercise-type dependent, and that the reductions in plasma levels of BA are more prolonged after RE than after EE, which is concurrent with our results. This divergent response is not surprising since EE and RE engage different types of muscle groups and type of fibres [37], resulting in an exercise-type specific metabolite fingerprint [38]. Nonetheless, these comparisons between type of exercise should be interpreted with caution since there were not matched in terms of intensity and duration. Further studies profiling the plasma levels of BA after longer recovery periods (i.e., 6, 12, or 24 h after EE and RE) are needed to determine when BA returns to their baseline levels.

Physiological significance of the reduction of plasma levels of bile acids after exercise

The physiological relevance and the mechanisms whereby exercise transiently reduces the circulating levels of BA are poorly understood. It is known that acute exercise increases systemic inflammation and oxidative stress [39]. For instance, a single bout of exercise increases the circulating levels of proinflammatory molecules and immune cells, such as IL-6 and neutrophils [40]. In fact, these increases in circulating inflammatory and immune markers after a single bout of exercise are actually linked to the health benefits of exercise [40]. However, circulating levels of BA follow an inverse direction and rapidly decrease after both EE and RE. One hypothesis for this phenomena involves a reduction of BA intestinal recycling or enterohepatic circulation as a consequence of a reduction of blood supply to the gastrointestinal tract. Indeed, many athletes usually report lower gastrointestinal symptoms such as abdominal pain and

diarrhoea while exercising , which suggests that mesenteric blood flow during intense exercise might be altered or reduced [41,42]. However, other studies has suggested that blood flow to the lower gastrointestinal tract remains unaltered or even increased during exercise [43–45]. A second scenario involves a reduction in hepatic BA production during exercise, since during this period there is no need of absorption of dietary lipids and fat-soluble vitamins, a process which is facilitated by BA production and secretion [6]. Further studies are warranted to determine if any of the aforementioned hypotheses or others could explain the acute decrease of circulating levels of BA in the first moments after finishing a bout of exercise and the mechanisms that control this complex process.

Impact of health status on plasma levels of bile acids after acute endurance exercise

Previous evidence has suggested that the different response of serum levels of BA in response to an oral glucose tolerance test (OGTT) between women with high and low CRF levels is linked to differences in insulin sensitivity status through mechanisms that might involve BA signaling. In a similar fashion that an OGTT, exercise constitutes a metabolic challenge that requires a fine regulation of glucose and insulin signalling pathways to meet its energetic demands [46]. Our findings revealed that individuals with higher CRF levels displayed a unique increase in plasma levels of CA (+77%) and CDCA (+65%) 120 min after EE. Remarkably, high CRF individuals presented better glucose parameters (-45% serum insulin levels, -49% HOMA index) than their counterparts, suggesting that the increase in CA and CDCA could be related to their improved glucose metabolism. This is congruent with preclinical studies in which the activation of the TGR5 in muscle and pancreas is linked to an improved glucose tolerance [47–49]. Interestingly, TGR5 is activated by several BA, and particularly the unconjugated forms of CA and CDCA are the most potent TGR5 activators among primary BA [50]. Specifically, the activation of TGR5 improves muscle function in mice, which concurs with explanation of the beneficial effects of exercise through BA signaling on skeletal muscle [48]. This is further supported by experiments in diabetics mice, where TGR5 agonism ameliorated insulin resistance in skeletal muscles through the activation of the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) and partially restored systemic glucose homeostasis [51]. Moreover, the muscle-specific TGR5 overexpression is sufficient for improving glucose metabolism in diet-induced obese mice in comparison to wild-type animals [49]. Altogether, it seems that after EE there is coordinate response to increase the production of unconjugated primary BA that leads to this particularly rise in plasma levels of CA and CDCA 120 min after EE. Moreover, this increase seems to be reflective of the better health status of high CRF individuals and might be related to a better glucose homeostasis via a BA-

TGR5 activation axis in skeletal muscle. Future mechanistic studies are needed to confirm this hypothesis.

Fecal microbiota composition seems to have no impact on plasma levels of bile acids after acute endurance exercise

The gut microbiota plays an important role in the metabolism of BA, and modulates its circulating concentrations through the expression of bile salt hydrolases (BSHs) and 7-α-dehydroxylases, the bacterial enzymes involved in BA metabolism [52]. BSHs participate in the deconjugation of primary and secondary BA by catalysing the removal of glycine and taurine [52], whereas 7-α-dehydroxylases enzymes convert primary BA to secondary BA [53,54]. It is known that athletes, which present higher CRF levels in comparison to sedentary individuals, also present a different gut microbiota composition [55,56] and it could constitute a critical component of their physical performance [57]. However, we found no differences in fecal microbiota composition between individuals with high and low CRF levels. Interestingly, it has been reported that BA profiles in response to different dietary challenges are not influenced by the fecal microbiota composition and diversity of individuals, but by variants in small intestinal BA transporter encoding genes [58]. Based on that, it might be possible that not only genes related to BA synthesis, but also genes involved in small intestinal BA transporter synthesis, could be explaining the specific BA signature in response to EE of the CRF individuals. Further research is warranted to unveil if gut microbiota composition might influence the acute exercise response of circulating BA in humans.

Strengths and limitations

Our study suffers from various limitations. Moreover, our findings may not be extrapolatable to trained and/or older individuals, individuals with metabolic complications that affect glucose metabolism (e.g., type 2 diabetes). We did not measure taurine-conjugated BA and neither the unconjugated form of LCA and UCDCA. Finally, the sex heterogeneity of our cohort precludes us to evaluate to what extent the response of plasma levels of BA to acute exercise could be sex dependent. On the other hand, our study presents several strengths. For instance, we provided a well-phenotyped cohort (e.g., body composition, cardiometabolic risk factors, BAT), and is the only one that has considered the potential role of fecal microbiota. Moreover, our study comprises both EE and RE, which offers a comparative overview of the effects of different types of exercise on plasma levels BA in humans

CONCLUSIONS

Our study reveals that a single bout of exercise rapidly decreases plasma levels of BA in an exercise-type specific manner in young, sedentary adults. Notably, individuals with higher CRF levels showed an increase in plasma levels of the primary BA CA and CDCA above their baseline levels at 120 min after EE. These increases in CA and CDCA after EE seems to be reflective of the better health status of these individuals in comparison to their low CRF levels counterparts and might be associated with a better or more adaptive response to exercise, independently of their fecal microbiota composition.

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RESULTS AND DISCUSSION

SECTION II

STUDY III: Activation of Human Brown Adipose Tissue by Capsinoids, Catechins, Ephedrine, and Other Dietary Components: A Systematic Review

***Osuna-Prieto FJ**, Martinez-Tellez B, Sanchez-Delgado G, Aguilera CM, Lozano-Sánchez J, Arráez-Román D, Segura-Carretero A, Ruiz JR. Activation of Human Brown Adipose Tissue by Capsinoids, Catechins, Ephedrine, and Other Dietary Components: A Systematic Review.*

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ABSTRACT

Human brown adipose tissue (BAT) has attracted clinical interest not only because it dissipates energy but also for its potential capacity to counteract obesity and related metabolic disorders (e.g., insulin resistance and dyslipidemia). Cold exposure is the most powerful stimulus for activating and recruiting BAT, and this stimulatory effect is mediated by the transient receptor potential (TRP) channels. BAT can also be activated by other receptors such as the G-protein-coupled bile acid receptor 1 (GPBAR1) or β-adrenergic receptors. Interestingly, these receptors also interact with several dietary components; in particular, capsinoids and tea catechins appear to mimic the effects of cold through a TRP-BAT axis, and they consequently seem to decrease body fat and improve metabolic blood parameters. This systematic review critically addresses the evidence behind the available human studies analyzing the effect of several dietary components (e.g., capsinoids, tea catechins, and ephedrine) on BAT activity. Even though the results of these studies are consistent with the outcomes of preclinical models, the lack of robust study designs makes it impossible to confirm the BAT-activation capacity of the specified dietary components. Further investigation into the effects of dietary components on BAT is warranted to clarify to what extent these components could serve as a powerful strategy to treat obesity and related metabolic disorders.

BACKGROUND

Brown adipose tissue (BAT) generates heat via non-shivering thermogenesis (NST) to keep core body temperature constant at low ambient temperatures [1–4]. Among other mechanisms [5], NST occurs by the action of the uncoupling protein 1 (UCP1), a molecular hallmark of BAT [6]. This protein is expressed in both brown adipocytes (classical BAT) and brite adipocytes (brown-like adipocytes emerging in white adipose depots, also known as beige adipocytes) [7]. The sympathetic nervous system (SNS) is the main regulator of BAT activity, realising norepinephrine through terminal neurons [8]. The surface of BAT adipocytes is rich in β-adrenergic receptors (β-ARs), which bind to norepinephrine [9]. These β-ARs are coupled to a Gs protein system that activates the enzyme adenylyl cyclase and lead to the formation of cyclic adenosine monophosphate (cAMP) as a second messenger [10]. cAMP activates protein kinase A, what leads to the activation of the thermogenic programme [9]. Both intra-cellular fatty acids and those coming from the blood stream are the main substrate of BAT mitochondria [11]. Circulating glucose is also a fuel for of brown adipocytes, allowing imaging techniques to use labelled glucose to trace human BAT activity [12]. However, a set of techniques based on lipid metabolism are being postulated as an alternative [13].

Several studies showed a negative association of human BAT activity and/or volume with body mass index (BMI) [14], fat mass [15–17], glucose levels [16,18], total cholesterol and triglycerides [19,20], and with the incidence of type II diabetes [21]. Thus, since its "re-discovery" in humans in 2009 [4,22–24], BAT has been postulated as a potential target tissue to face obesity and related diseases. Cold exposure is the main BAT activating stimulus [25]. It stimulates the transient potential receptor (TRP) channels that activate the SNS response [26]. Acute and/or chronic cold exposure effectively increases BAT volume and activity in humans, and improves overall metabolic health in healthy, obese, and diabetic patients [27–30]. However, cold acclimation is difficult to implement in clinical practice and is unpleasant for patients [26,31].

Interestingly, TRP channels not only mediate temperature stimuli, but are also chemesthetic receptors of substances naturally present in food and herbal plants [32]. TRP vanilloid 1 (TRPV1), TRP ankyrin 1 (TRPA1), and TRP melastin 8 (TRPM8) seem to be the most relevant for BAT activation, as their stimulation is associated with an increased BAT activity [33]. Currently, it is known that several thermogenic food ingredients (hereafter referred to as bioactive ingredients) are able to mimic cold exposure through the activation of TRP channels, consequently stimulating BAT [26]. The activation of TRPV1,TRPA1, and TRPM8 [34] could to be protective against obesity and cardiovascular risk preventing dietary-

induced body fat gain and inhibiting pro-inflammatory pathways [35–37]. In addition, some of these bioactive ingredients can increase cold-induced thermogenesis (CIT) as well as diet-induced thermogenesis (DIT) [38]. These effects might be partially explained by BAT, since DIT is higher in BAT-positive subjects compared to BAT-negative subjects [39] and the association between cold-induced BAT activation and CIT, independently of age and fat-free mass [40].

To date, there is increasing scientific evidence suggesting that bioactive ingredients may play a role in human BAT volume and activity [41]. We systematically reviewed the available human intervention studies analysing the effect of bioactive ingredients on BAT in order to know the potential clinical relevance of this promising strategy.

METHODS

We conducted a systematic search of articles of interest in PubMed and Web of Science . Our search strategy included articles from 1 January 2007, the year of the publication the first article suggesting that BAT was metabolically active in adult humans, until 1 February 2018 [42].

Search strategy

Search terms related to studies of brown fat in humans were combined in the following strategy in PubMed: (((((((“Adi- pose Tissue, Brown” [Mesh] OR “Brown Fat” OR “Brown adipose tissue”))) OR ((“Adipose tissue, beige” [Mesh] OR “beige adipose tissue” OR “Brite fat” OR “beige fat”))))) NOT ((((((((((“Mice” [Mesh]) OR “Rats” [Mesh]) OR “Animal Experimentation” [Mesh]) OR “Models, Animal” [Mesh])) OR (“rats” OR “mouse”))) OR “mice”)) OR “rat”))) NOT “Review” [Publication Type]; and in Web of Science: ((“Brown adipose tissue” OR “Brown fat” OR “Brite adipose” OR “Beige adipose” OR “Beige fat” OR “brite fat”) NOT (“Mice” OR Rat∗ OR (Experiment∗ AND Animal∗)OR (Research∗ AND Animal∗)OR “mouse” OR (model∗ AND animal∗))). File type: (ARTICLE OR CLINICAL TRIAL OR CASE REPORT

Study selection

The inclusion criteria were as follows: 1) bioactive ingredients (those components that met the bioactive ingredient definition criteria, that is any non-artificially synthetized/pharmaceutical chemical component of biological origin able to elucidate a significant thermogenic response when administrated orally or injected); 2) human studies; 3) original studies: no reviews; 4) articles written in the English language. Studies which included cancer reports such as pheochromocytoma or hibernoma were excluded. After having discarded the

duplicates found in both data bases, eligibility for inclusion was evaluated on the following: 1) reading title and abstract and 2) reading the full text.

Data extraction

The following data were collected from each included study: 1) dietary compound; 2) source; 3) dose; 4) year; 5) country; 6) season/month; 7) study design; 8) BMI; 9) participants' sex; 10) main findings; 11) BAT measurement technique; 12) ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography (PET) combined with computed tomography (CT) (^{18}F-FDG PET/CT) measurement point; 13) reference.

RESULTS

General results

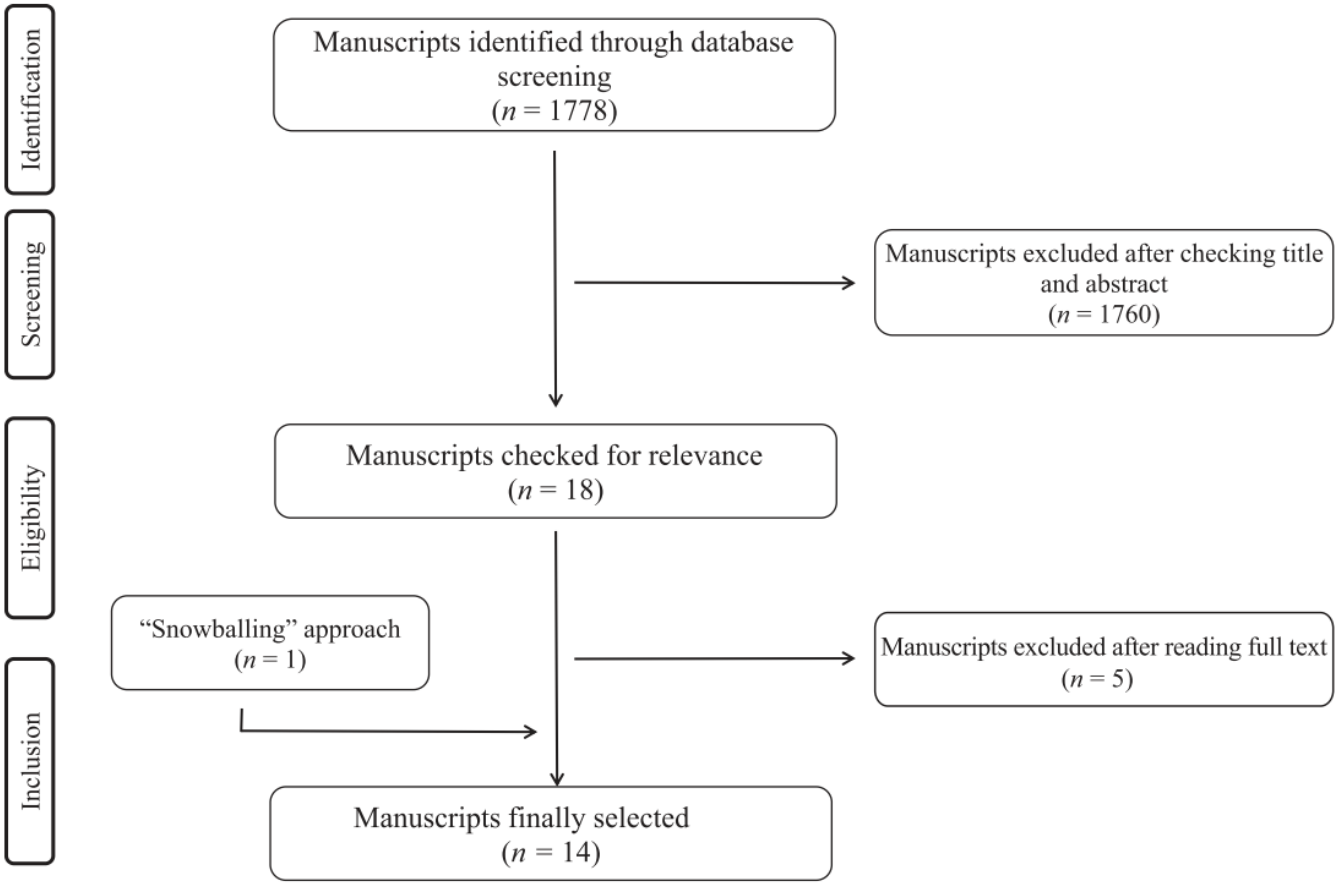
A total of 1778 studies were identified after duplicates were discarded (**Fig. 1**). No additional information was retrieved after repeating the search in Scopus (information not included in the flowchart). A total of 14 manuscripts were finally included after applying the inclusion and exclusion criteria. Two of the manuscript [43,44] included 2 studies in the same manuscript, resulting a total of 16 studies. We found no studies conducted in participants with metabolic syndrome or type II diabetes. Notably, some studies did not report information regarding the season in which the study was conducted [45–47], whereas others were conducted completely [40,43,44,48–50] or partially [51–53] in winter. Due to the high heterogeneity of the identified studies, no quality-assessment scale systems were used to evaluate the quality of our eligible studies.

The most-studied bioactive ingredients were capsinoids (n = 6 studies) [40,43,48,49,54], followed by tea catechins (n = 3 studies) [44,50] and ephedrine (n = 3 studies) [45,52,55]. Other studies focused on bile acids and different plant and seaweed extracts [46,47,53,56]. All studies were published between 2012 and 2018. The Japanese group headed by Dr. Saito conducted nearly half of the studies in this field (n = 6; 35%) [40,44,46–48]. A total of n = 12 (75%) of the studies were performed in Asians [40,43,44,46–50,54,56] and 25 % (n = 4) [45,52,53,55] were conducted in Caucasians. Notably, only 3 [43,52,56] out of the 16 studies conducted ^{18}F-FDG PET/CT scans before and after the intervention **(Table 1).** Another 3 studies (19%) performed the ^{18}F-FDG PET/CT scan only after the intervention [45,53,55]. A total of 13 (81%) studies used the ^{18}F-FDG PET/CT scan to quantify BAT activity and/or volume before and/or after the intervention (**Table 1**). Two studies [43,50] (n = 2; 12,5%) used near-infrared time-resolved spectroscopy (NIR_{TRS}), whereas only one study used infrared thermography (IRT) (n = 1; 6%) [49] to quantify BAT activity. No

study conducted biopsies. All studies were conducted in adults under the age of 35 y [40,43,53,55,56,44–50,52], 14 studies were conducted in healthy and lean humans [40,43,55,44,46–50,52,53] and 2 were conducted in obese humans [45,56].

Figure 1. Flowchart showing the literature search and article selection process.

Capsinoids



All studies on capsinoids used the oral administration of 9 mg capsinoids/d extracted from *Capsicum annum L.* (CH-19 sweet chili pepper), except for a single study [54] that administrated 12 mg/d (acute effect). There were three chronic-effect studies that used 9 mg capsinoids/d capsinoids (between 6 and 8 weeks) [40,43] and three acute-effect studies [48,49]. Yoneshiro et al. [40] conducted a chronic-effect study (**Table 1**) in which the participants were selected according to their BAT activity. After 6 wk of capsinoid treatment, the participants who had received capsinoids exhibited a significant increment in CIT capacity compared with the control group [40]. Nirengi et al. [43] reported that 6 wk of capsinoid treatment induced an increase in BAT activity measured by ^{18}F-FDG PET/CT scan [43]. The same group [43] studied the effect of a capsinoid ingestion over 8 wk and showed that total haemoglobin (total-Hb) change (assessed by NIR_{TRS} every two weeks at 27°C in the supraclavicular region) was significantly greater in the

capsinoids group [43]. Regarding acute-effect studies, Yoneshiro et al. [48] reported that acute-effect ingestion of capsinoids significantly increased resting metabolic rate (RMR) and supraclavicular skin temperature in the high-BAT activity group [48]. The observed increase in RMR was associated with an increase in BAT activity [48]. Moreover, Ang et al. [49] concluded that a single ingestion of capsinoids elicited a significant increase in RMR and in skin temperature in the cervical-supraclavicular region measured by infrared thermography in the capsinoid group [49]. Finally, Sun et al. [54] used the highest dose of capsinoids (12 mg/d) in healthy adults and compared this treatment with mild cold exposure: no effect of capsinoid ingestion on BAT stimulation was observed, whereas a mild cold exposure did stimulate BAT [54].

Tea catechins

All studies on tea catechins used green tea extract beverages from *Camellia Sinensis*, and 2 of these studies catechin also used caffeine supplementation [44]. Nirengi et al. [50] administrated a beverage containing 540 mg of catechins plus 77 mg of caffeine/d daily for 12 wk, and demonstrated an increase in BAT density evaluated by NIR_{TRS} [50]. Yoneshiro et al. [44] administrated a beverage containing 615 mg catechins plus 77 mg of caffeine twice daily for 5 wk. BAT activity was measured by ^{18}F-FDG PET/CT scan before the intervention with a previously fixed cooling protocol (2 h; 19^{o}C) to select participants with low BAT activity [44]. The authors demonstrated an increase in CIT in the catechin group relative to the control group [44]. The results of the experimental group [44] also revealed that a single ingestion of the beverage containing catechins plus caffeine induced a significant increase in RMR in the catechin group compared with the placebo group [44].

Ephedrine

The only available chronic effect study determined that BAT activity was significantly reduced by a 28-d ephedrine treatment (1.5 mg ephedrine hydrochloride $kg^{-1} \cdot d^{-1}$) in the experimental group compared with the control group [52]. The findings of the acute-effect studies are contradictory. Carey et al. [45] measured BAT activity 60 min after ingestion of 2.5 mg ephedrine/kg, and revealed an increase in BAT activity in lean but not in obese humans. In contrast, Cypess et al. [55] detected no increase in BAT activity after an intramuscular injection of 1mg ephedrine/kg.

Table 1.Summary of studies found in the systematic review that reported and increment/decrement in BAT activity after the ingestion or administration of a bioactive ingredient.

Bioactive ingredient	Source	Dose	Year	Country	Season or month	Study design	BMI (kg/m²)	n	Age (years)	Sex	Main findings	BAT activity measurement technique	^{18}F-FDG PET/CT Measurement point	Ref.
Capsinoids	CH-19 Sweet (*Capsicum annuum L.*)	9 mg (acute-effect)	2012	Japan	January-March (Winter)	single-blind, randomised, placebo-controlled, crossover	21.3 ± 0.4	18	22.8 ± 0.7	male	RMR significantly increased by 15.2 ± 2.6 kJ/h in 1 h in the BAT-positive group and by 1.7 6 3.8 kJ/h in the BAT-negative group after oral ingestion of capsinoids	^{18}F-FDG PET/CT	before administration	[48]
Capsinoids	CH-19 Sweet (*Capsicum annuum L.*)	9 mg/day for 6 weeks	2013	Japan	January-March (Winter)	single-blind, randomised, placebo-controlled, crossover	22.0 ± 0.4	10	24.4 ± 0.5	male	CIT after capsinoid treatment (200.0 ± 33.9 kcal/d) was significantly higher than before capsinoid treatment (20.6 ± 43.0 kcal/d) and after placebo treatment (81.0 ± 32.5 kcal/d	^{18}F-FDG PET/CT	before administration	[40]
Capsinoids	CH-19 Sweet (*Capsicum annuum L.*)	9 mg/day for 6 weeks	2016	Japan	December-March (Winter)	single-blind, crossover	Not reported	3	Not reported	male	SUVmax increased by 48.8% (2.2 ± 0.3 before vs. 3±1.3 after) after capsinoid ingestion	^{18}F-FDG PET/CT	before & after administration	[43]

Capsinoids	CH-19 Sweet (*Capsicum annuum L.*)	9 mg/day for 8 weeks	2016	Japan	January-March (Winter)	double-blind, randomised, placebo-controlled, crossover	21.7 ± 1.4	20	20.8 ± 1.1	male & female	Supraclavicular [total-Hb] increased by 46.4% after the capsinoids treatment (70.4 ± 14.8 before vs. 102.2 ± 27.2 μM after)	NIR_{TRS}	No measurement	[43]
Capsinoids	CH-19 Sweet (*Capsicum annuum L.*)	9 mg (acute-effect)	2016	Japan	October-June (autumn to summer)	double-blind, randomised, placebo-controlled, crossover	20.4 ± 0.3	24	23 ± 0.4	male	RMR was significantly higher (66.90%) in subjects who had ingested capsinoids vs. placebo in the high-BAT group	IRT	No measurement	[49]
Capsinoids	CH-19 Sweet (*Capsicum annuum L.*)	12 mg (acute effect)	2018	Japan	August-March (summer to winter)	Non-blind, crossover	21.7 ± 0.6	20	25.9 ± 0.9	male & female	Capsinoids did not stimulate BAT above the PET SUV-threshold of 2.0 SUVmean, whereas mild cold did	^{18}F-FDG PET/CT	after administration	[54]
Tea catechins	green tea extract (*Camellia sinensis)*	540 mg/day for 12 weeks	2016	Japan	December-March (winter)	double-blind, randomised, placebo-controlled crossover	21.1 ± 1.5	21	20.8 ± 2.1	female	There was a significant increase in [total- Hb] after 12 weeks of catechin beverage ingestion (67.9 ± 20.4 before vs. 80.6 ± 24.3 μM after)	NIR_{TRS}	no measurement	[50]
Tea catechins & caffeine	green tea extract (*Camellia sinensis*)	615 mg tea catechins and 77 mg caffeine (acute effect)	2017	Japan	January-March (winter)	single-blind, randomised, placebo-controlled, season-matched crossover	21.4 ± 0.7	15	23.1 ± 0.6	male	RMR was significantly higher after the ingestion of the catechin beverage than the placebo at 0–90 and 0–180 min (quantification of AUC expressed as figures not reported)	^{18}F-FDG PET/CT	before administration	[44]

Tea catechins & caffeine	green tea extract (*Camellia sinensis*)	615 mg tea catechins + 77 mg caffeine for 5 weeks	2017	Japan	January-March (winter)	single-blind, randomised, placebo-controlled, season-matched crossover design	20.7 ± 0.5	20	22.7 ± 0.7	male	CI fat oxidation was significantly higher after the treatment with the catechin beverage (33.7 ± 4.8 mg/min) than before the treatment (22.8 ± 3.8 mg/min) and also significantly higher after the placebo (15.2 ± 5.8mg/min)	^{18}F-FDG PET/CT	before administration	[44]
Ephedrine	*ephedra* extract	2.5 mg/kg (acute effect)	2012	Australia	Not reported	double-blind, randomised, placebo-controlled, crossover	22 ± 1 (lean) 36 ± 1 (obese)	18	25 ± 1 (lean) 27± 1 (obese)	male	There was a significant increase in supraclavicular SUVmax by 145 ± 4 8% in lean, but not in obese participants (4.0 ± 8.3%)	^{18}F-FDG PET/CT	after administration	[45]
Ephedrine	*ephedra* extract	1 mg/kg (acute effect)	2012	United States	February-March (throughout the year)	double-blind, randomised, placebo-controlled	23.7 ± 1.4	10	27.1 ± 1.7	male & female	Ephedrine treatment did not reach a measurable effect on detection of BAT activity, whereas cold exposure did	^{18}F-FDG PET/CT	after administration	[55]
Ephedrine	*ephedra* extract	1.5 mg/kg/day for 4 weeks	2015	Australia	March-October (autumn to spring)	double-blind, randomised, placebo-controlled	24.5 ± 1.5	23	22.5 ± 1.5	male	Both SUVmax (placebo: −3 ± 6%; ephedrine: −13 ±7 %) and SUVmean values (−3±7%; ephedrine −22 ±6 %) decreased after the ephedrine treatment	^{18}F-FDG PET/CT	before & after administration	[52]

6-paradol, 6-gingerol & 6-shogaol	Grains of Paradise *(Aframomum melegueta)*	40 mg (acute effect)	2013	Japan	Not reported	single-blind, randomised, placebo-controlled, crossover	21.3 ± 0.6	19	22.6 ± 0.7	male	EE response to extract ingestion during the 2h period expressed as AUC was 24.7 ±6.1 kJ/h in the BAT-positive group, which was significantly higher than in the BAT-negative group (23.2 ± 10.1)	^{18}F-FDG PET/CT	before administration	46
3,5,7,4′-tetramethoxy-flavone, dimethoxy-flavone & pentame-thoxyflavone	*Kaempferia parviflora (KP)* extract	100 mg (acute effect)	2014	Japan	Not reported	single-blind, randomised, placebo-controlled crossover	21.2 ± 0.3	20	24.1 ± 0.4	male	An increase in EE after the ingestion was observed in the high-BAT group (351±50 kJ/d at 60 min), but not in the low-BAT activity group	^{18}F-FDG PET/CT	before administration	47
xanthigen (fucoxanthin + punicic acid)	*Undaria pinnatifida*	600 mg fucoxanthin for 3 months	2015	Korea	August-November (summer to autumn)	pilot study	36.6	2	32.5	female	Xanthigen visually increased BAT activity in one of the two participants.	^{18}F-FDG PET/CT	before &after administration	56
Chenodeoxycholic acid	Synthetic production	15 mg/kg for 2 days (acute effect)	2015	The Netherlands	February-June (winter to spring)	double-blinded, randomised, placebo-controlled crossover	21.9 ± 1.6	12	22 ± 3	female	Chenodeoxycholic acid increased both BAT SUVmean (from 0.8 ± 0.3 to 1.2 ± 0.3) and SUV max values (from 1.0 ± 0.4 to 1.6 ± 0.4)	^{18}F-FDG PET/CT	after administration	53

BMI and age values are means ± SD. AUC: area under the curve; BAT: brown adipose tissue; BMI: body mass index; CI: cold induced; CIT: cold induced thermogenesis; EE: energy expenditure; F-FDG PET/CT: fluorodeoxyglucose positron emission tomography/computed tomography; Hb: haemoglobin ; IRT: infrared thermography ; N: sample size; NIR_{TRS}: near-infrared time-resolved spectroscopy; RMR: resting metabolic rate SUVmax: maximum standardized uptake value;TRPA1: transient receptor potential cation channel ankyrin 1; TRPV1: transient receptor potential cation channel vanilloid receptor 1; β3-AR: beta-3 adrenergic receptor.

Other bioactive ingredients

Three studies used plant extracts with pungent activity [46,47,56], and 1 study used bile acids [53]. Only 1 study evaluated the chronic-effect of the bioactive ingredient [56] and 3 studies evaluated the acute-effects [46,47,53].

One study used a seaweed extract containing fucoxanthin on 2 obese adult women [56]. The women were instructed to take 2 pills of Xanthigen (600 mg contained in 2 capsules; PLT Health Solutions) with fucoxanthin (3mg) and punicic acid (174mg) every day for 12 wk. The ^{18}F-FDG PET/CT scan analyses, which were conducted before and after the 12-wk intervention, reported a visual (not quantitative) increase in BAT activity in 1 of the participants. Sugita et al. [46] used a single dose of 40mg from *Aframomum melegueta*. The participants were divided into BAT-positive and negative groups. After the oral ingestion of the bioactive ingredients, a significant increase of RMR was found in the BAT positive group [46]. Matsushita et al. [47] used a single dose 100 mg of *Kaempferia parviflora* extract. All participants were men and were previously divided into high-BAT-activity and low-BAT-activity groups. A significant increase in RMR in the high-BAT was demonstrated. The acute effect of chenodeoxycholic acid (15 mg $\cdot$ kg^{-1} $\cdot$d^{-1} for 2 d) was tested in young and lean women [53]. They reported a significant increase in BAT activity and an increase in RMR in the chenodeoxycholic acid group [53].

DISCUSSION

We analysed all available human studies that investigated the effect of both chronic and acute ingestion of bioactive ingredients on BAT activity, as measured by ^{18}F-FDG PET/CT, and on CIT, RMR, supraclavicular total-Hb, and supraclavicular skin temperature. In general, the study designs were not robust, because only 7 (47%) [43,45,49,50,52,53,55] used a double-blind, randomized, placebo-controlled design, and only 3 studies [43,52,56] conducted an ^{18}F-FDG PET/CT scan before and after the intervention. There is a lack of information on the effect of the bioactive ingredients at the molecular level in BAT, white adipose tissue, or muscle measured *in vivo* through biopsy analysis. The results of several studies [40,43,54,56,44–50,53] suggest that it seems plausible to activate and recruit human BAT through the ingestion of certain bioactive ingredients in healthy adults, yet the current level of evidence precludes a definitive conclusion. Further studies are warranted to confirm this hypothesis.

Capsinoids

Capsinoids are substances naturally present in chilli peppers, and they are particularly abundant in *Capsicum annum L.* or "CH-19 sweet" [57]. Capsinoids include capsiate, dihydrocapsiate, and nordihydrocapsiate [58]. Although capsinoids are structurally similar to capsaicin, they are 1/1000 less pungent but as potent as capsaicin tin increasing thermogenesis and RMR [48].

The thermogenic activation pathways of capsinoids include TRPV1 and TRPA1 which have possible mechanisms of action on BAT activity, because capsinoids activate both receptors [59,60]. In mice, intragastric administration of capsinoids has shown to elicit an increase in temperature in the intrascapular BAT region, whereas this effect was attenuated in TRPV1 deficient animals [61]. The thermogenic response is also impaired in humans with a mutation affecting the TRPV1 function [62]. Furthermore, capsiate is an enhancer of the UCP1 expression [63]. Consequently, it is likely that capsinoids activate BAT through the TRPV1-SNS-BAT axis in humans. Only 1 study on capsinoids analysed BAT activity before and after the intake of bioactive ingredients [43] and only one before the capsinoids ingestion. Notably, the first study [43] had a low sample size (n = 3; single-blind and crossover study design), whereas the second study [54] had a non-blind design. Neither study [43,54] performed a personalised cooling protocol before the ^{18}F-FDG PET/CT scan, and the glucose standardized uptake values were not individualised [43] and did not meet the recommendations of BAT analysis and quantification [64].

Tea catechins

Tea catechins are polyphenolic components present in green tea. The most abundant and bioactive component is the epigallocatechin gallate, which is one the most thoroughly studied bioactive ingredient present in green tea. Therefore, epigallocatechin gallate may be the best choice if only 1 catechin is encapsulated to test its properties on BAT activity. The thermogenic effect of tea catechins has repeatedly been shown in humans [65,66]. Regarding the mechanism of action, epigallocatechin gallate and its auto-oxidation products have shown to be TRPA1 and TRPV1 agonists [67,68]. Catechins can activate and recruit BAT via TRP channels located in the sensory neurons of the gastrointestinal tract [69]. There is no solid evidence that tea catechins can activate and recruit BAT in humans, because there are no studies in which ^{18}F-FDG PET/CT scans were conducted before and after the bioactive ingredient administration. However, it seems biologically plausible that tea catechins plus caffeine could activate human BAT in both chronic and acute treatments, because this combination has shown to increase CIT and RMR in humans [44]. Certain studies suggest that CIT may be

proportional to BAT-dependent thermogenic capacity [70]. Therefore, the significant increase in CIT after the tea catechin treatment could be due to an increase in BAT activity. Interestingly, there is strong evidence that skeletal muscle is as a major contributor of CIT, so we cannot discard the possibility that the effects of catechins are muscle-mediated and not BAT-mediated [71,72]. Nirengi et al. [50] quantified the change in total- Hb in the supraclavicular region after a chronic intake of catechins under thermoneutral conditions, which was found to be significantly higher in the catechin group[50]. This finding is in agreement with the results of a previous experiment performed by the same research group in which they demonstrated that total-Hb values under thermoneutral conditions were positively correlated with BAT activity [73]. Thus, it seems feasible that the increase in total-Hb in the supraclavicular area may be directly correlated with an increase in BAT activity, and that tea catechins could activate BAT even under thermoneutral conditions in healthy humans

An acute-effect study [44] showed an increase in RMR after the ingestion of a tea catechin beverage in subjects with detectable BAT activity but not in subjects with undetectable BAT activity. Even though this increase in RMR was likely due to an increase in BAT activity, no ^{18}F-FDG PET/CT scan was conducted after the intervention to confirm the finding.

In vitro studies using tea catechins have revealed an inhibition of the catecholamine-degrading enzyme catechol-O-methyltransferase [74], which could explain the SNS- BAT connection as due to an increase in norepinephrine life span. Nonetheless, catechol-O-methyltransferase activity was not impaired by high doses of epigallocatechin gallate in humans [75]. Regarding the thermogenic effects of caffeine, these effects may occur through the inhibition of phosphodiesterase (an enzyme that degrades cAMP) [76]. In addition, a synergistic interaction has been proposed between tea catechins and caffeine, with the latter increasing adrenergic and lipolysis activity [77,78]. Additional studies are warranted to clarify whether the inhibition of catechol-O- methyltransferase and phosphodiesterase is responsible, in part, for the thermogenic effect of tea catechins and to verify to what extent TRPA1 and TRPV1 activation are able to enhance human BAT activity.

Ephedrine

The bioactive ingredient ephedrine is a sympathomimetic amine found in plants of the Ephedra genus, which can bind to adrenergic receptors. The mechanism of action of ephedrine does not involve activation of the TRP receptor, but rather, stimulation of SNS activity and thermogenic pathways boosts BAT activity. Historically, ephedrine has been used to increase energy expenditure in humans [79], and it has been linked to an increase in ^{18}FDG-BAT uptake in mice [80]; thus it

seems plausible that ephedrine itself could activate BAT in humans. Carey et al. [45] showed that BAT can be activated in healthy, lean adults with a single dose of ephedrine. Interestingly, the same treatment administered to obese patients did not significantly increase BAT activity, suggesting that, at least in response to sympathomimetic bioactive ingredients, BAT activity may be impaired in obese humans. This finding is consistent with previous cold exposure studies that did not detect a significant increase in BAT in obese humans [4,15,24]. Conversely, another acute-effect study performed by Cypess et al. [55] failed to stimulate BAT activity after ephedrine administration. However, this study used a single intramuscular dose of 1 mg ephedrine/kg, which was a lower dose (and a different route of administration) than that used by Carey et al. [45]; hence, we cannot discern to what extent the difference between intramuscular injection and oral ingestion affected the outcome. Moreover, the analyses were performed over a wide period of time (>1 y considering all the interventions and measurements of all participants) so the seasonal variations in BAT activity could have introduced a bias.

Chronic effects of ephedrine treatments appear to reduce BAT activity. Carey et al. [52] showed that BAT activity was reduced after 28 d of ephedrine treatment. Interestingly, this intervention was performed from spring to autumn, far from the winter season, which means that the warmer outdoor temperatures might have also inhibited BAT activity [29]. Further studies are needed to determine whether BAT activation could be due to ephedrine itself, what the ideal dose and via of administration (intramuscular or orally ingested) are, and to what extent the duration of the treatment impairs BAT activity.

Other bioactive ingredients

A. melegueta seeds, also known as "Grains of Paradise," are used as a spice for flavouring food and are known to have anti-inflammatory properties [81]. These seeds are rich in 6-gingerol, 6-paradol, and 6-shogaol (all of which are non-volatile with pungent activity) [82]. It seems feasible that these bioactive ingredients may exert their effects by binding TRPV1 [69]. Sugita et al. [46] demonstrated an increase in RMR in BAT-positive individuals compared with BAT-negative individuals after am intervention with the *A. melegueta* [46]. However, the effect of *A. melegueta* on BAT activity was not tested and is therefore unknown because the ^{18}F-FDGPET/CT scan was performed only before the intervention. The volatile components existing in the A. melegueta extract have a vanilloid moiety, which can activate TRPV1 (involved in the thermic effects of capsinoids and catechins, as previously described). Therefore, if the increase in RMR after the ingestion of *A. melegueta* is confirmed by a parallel augmentation in BAT activity

assessed after ingestion of the bioactive ingredient, then the activation of the TRPV1- SNS-BAT axis may be the underlying mechanism of action.

Regarding human BAT activity after oral ingestion of the *K. parviflora* extract, RMR increased in the BAT-positive group [46]. *K. parviflora has been demonstrated to have anti-obesity effects in type 2 diabetic obese mice* [83]. Dietary supplementation with K. parviflora prevented not only body weight increase and body fat accumulation but also glucose intolerance [83,84]. Yoshino et al. [85] showed that K. parviflora ingestion increased urinary excretion of noradrenaline, UCP1 expression, and RMR in mice. Thus, it could be expected that K. parviflora, similar to capsinoids, activates and recruits BAT. Nevertheless, studies with better methodological designs are warranted to determine whether K. parviflora truly enhances BAT activity in humans.

Xanthigen is a weight-management ingredient combining punicic acid (from pomegranate) and fucoxanthin from the brown edible seaweed Undaria pinnatifida. The combination of Xanthigen with punicic acid appears to have positive effects on weight loss, body fat, and liver fat content in obese nondiabetic women [86]. In addition, fucoxanthin from U. pinnatifida has exhibited an anti-obesity effect through the enhancement of UCP1 expression in murine white adipose tissue [86]. Although. Kim et al. [56] performed a before- and-after assessment of BAT, the sample size of their study (n = 2) precludes any firm conclusion. Moreover, these authors reported a visual increase (not quantified) in BAT activity according to PET/CT (55). [56]. Thus, even though the results appear to support the evidence of previous studies demonstrating that Xanthigen increases RMR in obese patients [87] and could therefore be useful as a therapy against diabetes [88], a larger sample size and a better study design are needed.

Chenodeoxycholic acid is one of the primary bile acids produced by the liver in humans, and its supplemental use has been demonstrated to be safe in humans easily administered [89]. Chenodeoxycholic acid activates the G-protein–coupled bile acid receptor 1 (GPBAR1), which results in an increase in the concentration of intracellular cAMP. This secondary messenger, cAMP, activates type 2 deiodinase, an enzyme that drives the conversion of the inactive thyroid hormone to the active form (T3). Thus, T3 is the final activator of BAT, which also increases RMR [90]. It also appears to increase BAT activity and enhance RMR in murine models [91]. Moreover, there is strong evidence supporting a correlation between circulating bile acids, BAT activity, and NST [90]. Broeders et al. [53] showed that an administration of 15 mg chenodeoxycholic acid/d for 2 d increases BAT activity measured by ^{18}F-FDG PET/CT scan under thermoneutral conditions.

Therefore, it would be of clinical interest to study the effects of chronic chenodeoxycholic acid supplementation on BAT activity.

General limitations of the studies included in the review

Homogeneous composition of bioactive ingredient extracts

To enable interstudy comparisons, there is a need to standardize the use of bioactive ingredient extracts. For example, Yoneshiro et al. [44] and Nirengi et al.[50] used different concentrations of catechins in their beverages; therefore, it is not possible to determine to what extent the results between these studies are comparable.

^{18}F-FDG limitations

Even though 18F-FDG uptake is a marker of BAT activity, this parameter is not directly proportional to energy consumption, because glucose is not the major fuel in BAT metabolism [92]. Indeed, fatty acids, which are the main substrate of BAT mitochondria, are mostly provided from the inner depots, but are also provided from the bloodstream by the action of the lipoprotein lipase [12]. Hence, we should use additional approaches to quantify total BAT activity, such as oxygen consumption [71], tracking other fatty acid derivatives such as ^{11}C-acetate [93], and using MRI [94–97].

Ethnicity

There is evidence that BAT volume is dependent on ethnicity [98]. The majority of the studies included in the review were conducted in south Asians (75%; n = 12) [40,43,44,46–50,56] compared with 25% in Caucasians (n = 4) [45,52,53,55]. Therefore, caution must be used when translating the results from one ethnic group to another.

Seasonality

Seasonal changes in BAT and CIT could be due to some environmental factors such as outdoors temperatures [99] and photoperiod [100]. In addition, not only CIT but also cold-induced fat oxidation have shown to be greater in winter compared with summer, and this change is more notable in high-BAT subjects than in low-BAT subjects [101]. Considering all this evidence, the involvement of BAT in the seasonal variations of CIT in healthy humans is another variable warrants consideration to optimize study designs (*i.e.*, crossover with wash-out for acute-effect studies; control group with placebo for chronic-effects studies).

Dose adjustment

Only chenodeoxycholic acid [53] and ephedrine studies [45,52,55] adjusted the bioactive ingredient dose according to the weight of each participant.

Authorship

Notably, all studies using capsinoids and catechins were conducted by Japanese researcher groups [40,43,44,46–50], with Dr. Saito leading a significant fraction of the studies using bioactive ingredients (n = 6; 35%) [40,44,46–48]. To date, there is no confirmation of the results by an independent research group, except for Sun et al. [54] who studies capsinoids.

CONCLUSIONS

Although it is biologically plausible that the ingestion of food ingredients increases human BAT activity, the current level of evidence supporting this hypothesis is low. More and better-designed studies (e.g., double-blind, randomized, placebo-controlled, and season-matched, with a personalized cooling protocol prior to PET/CT or MRI scan) are needed to understand whether bioactive ingredients are an effective treatment to activate and recruit human BAT.

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RESULTS AND DISCUSSION

SECTION II

STUDY IV: Activation of Brown Adipose Tissue and Promotion of White Adipose Tissue Browning by Plant-based Dietary Components in Rodents: A Systematic Review

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ABSTRACT

Activation of brown adipose tissue (BAT) and promotion of white adipose tissue (WAT) browning is considered a potential tool to combat obesity and cardiometabolic disorders. The use of plant-based dietary components has become one of the most used strategies for activating BAT and promoting WAT browning in rodents. The main reason is because plant-based dietary components are usually recognized as safe when the dose is properly adjusted, and they can easily be administrated by being added to the diet or dissolved in water. The present systematic review aimed to study the effects of plant-based dietary components on activation of BAT and promotion of WAT browning in rodents. A systematic search of PubMed and Scopus (from 1978 to 2019) identified eligible studies. Studies assessing the effects of plant-based dietary components added to diet and/or water on uncoupling protein 1 (UCP1) expression in BAT and/or WAT were included. Studies that used dietary components of animal origin, did not specify the effects on UCP1, or were conducted in other species different from mice or rats were excluded. Of 3919 studies identified in the initial screening, 146 studies were finally included in the review. We found that tea extract catechins, resveratrol, capsaicin and capsinoids, cacao extract flavanols, and quercetin were the most studied components. Scientific evidence suggests that some of these dietary components activate BAT and promote WAT browning via activation of the AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1) pathways. These findings reveal that there is strong scientific evidence supporting the use of plant-based dietary components to activate BAT and promote WAT browning in rodents and thus to potentially combat obesity and cardiometabolic disorders.

BACKGROUND

Obesity is a global epidemic that increases the risk of morbidity and reduces lifespan, being closely related to an increased risk of developing cardiometabolic disorders [1,2]. Brown adipose tissue (BAT) is considered a target tissue to combat obesity [3] and cardiovascular disease (CVD) [4], as BAT activation increases energy expenditure, reduces adiposity, and effectively protects against diet-induced obesity in mice [5,6]. The thermogenic capacity of BAT is driven by uncoupling protein 1 (UCP1) activity, located in the inner mitochondrial membrane of brown adipocytes [7]. Interestingly, UCP1 can also be expressed in beige adipocytes (brown-like adipocytes within white adipose tissue, WAT), a process known as WAT browning [8]. The resulting increase in energy expenditure due to BAT activation goes beyond heat generation, improving glucose and lipid metabolism [9–11]. Actually, it seems that BAT also exerts an endocrine function through the so-called "batokines" (adipokines released by brown adipocytes) which could in part be responsible for the observed improvements in metabolism [12,13]. The existence of UCP1-independent thermogenic mechanisms is also known, yet their relevance in terms of energy expenditure remains poorly understood [14].

Cold exposure is the canonical stimulus for BAT activation [14], this being primarily mediated through beta-3 adrenergic receptor (β3-AR) stimulation in rodents [15]. Nevertheless, there are other ways to stimulate BAT activation and promote WAT browning (both understood as an increase in UCP1 expression), such as the pharmacologic agonism of the β3-ARs [16] and the glucagon-like peptide 1 (GLP1) receptor [17]. Increasing evidence suggests that plant-based dietary components, which can easily be added to the diet or dissolved in water [18], can also boost BAT activation and promote WAT browning [19–21]. Moreover, a significant fraction of these dietary components is generally recognized as safe (GRAS) in The United States, which results in a large list of potential candidates. This could explain, at least in part, the massive increase in the number of publications on this topic.

Therefore, the main goal of the present systematic review is to study the effects of plant-based dietary components on BAT activation and promotion of WAT browning in rodents.

METHODS

This systematic review was conducted following the Preferred Reporting Items for Systematic Reviews and Metanalysis (PRISMA) statement [22].

Inclusion and exclusion criteria

The inclusion criteria were as follows: 1) meeting the definition of plant-based dietary components: natural occurring isolated dietary component, selected isolated fraction of plant extracts or whole plant extracts of vegetal origin, and thus excluding those of animal origin (*i.e.,* conjugated linoleic, fish oil, or omega-3 fatty acids from animal origin); 2) administration: orally via mixture with diet or dissolved in water; 3) type of rodent: mice and rats; 4) UCP1 expression: measurements of UCP1 gene/protein expression in BAT or WAT; 5) original papers (not reviews); and 6) articles written in the English language.

The exclusion criteria were studies that: 1) included dietary components categorized as toxic (*i.e.*, alcohol or ephedrine); 2) included a control group with a different type of diet from the intervention group; 3) included housed animals at different temperatures from the intervention group. Eligibility for inclusion and exclusion criteria was evaluated by reading 1) title and abstract (n = 3,919), and 2) full text (when the information provided in the title and abstract was not enough to make an inclusion or exclusion decision) (approximately 300 publications).

Data collection process

The following data were extracted from each included study depicted in **Supplemental Table 2**: 1) plant-based dietary component; 2) daily dose (single dose for acute studies); 3) species (sex); 4) age (at the beginning of the intervention, weeks); 5) time of intervention with the plant-based dietary component (weeks); 6) housing temperature (^{o} Celsius); 7) light cycle (lights on: light off); 8) humidity (%); 9) food and water access (e.g. *ad libitum* or time-restricted); 10) type of diet 11); sample size (intervention group); 12) activation of BAT and promotion of WAT browning, studies that measured UCP1 at gene (qRT-PCR) and/or protein (immunoblot and immunostaining assays) expression levels in one or both tissues; 13) reference.

Search strategy

We employed three different term resources from the National Library of Medicine (NLM) controlled vocabulary thesaurus used for indexing articles for PubMed in our search: i) Medical Subject Headings (MeSH) terms: used for ceiling the search to publications where that term is the major focus of the content of the article; ii) Text Words (TW): it includes all words and numbers in the title, abstract, MeSH terms, MeSH Subheadings, publication types, and other relevant sections; iii) Supplementary concept: it includes chemical or organism-specific indexed terms. Search terms related to the main goal of the current systematic

review were combined using the following strategy in PubMed: ("Adipose Tissue, Brown"[Mesh] OR "Adipocytes, Brown"[Mesh] OR "browning"[tw] OR "beigeing"[tw] OR (("brown"[tw] OR "beige"[tw] OR "brite"[tw]) AND ("fat"[tw] OR "adipose"[tw] OR "adipocyte"[tw] OR "adipocytes"[tw] OR "thermogenesis"[tw])) OR "Uncoupling Protein 1"[Mesh] OR "Uncoupling Protein 1"[tw] OR "UCP1"[tw] OR "Ucp1 protein, rat" [Supplementary Concept] OR "Ucp1 protein, mouse" [Supplementary Concept]) AND ("Food"[Mesh] OR "food"[tw] OR "foods"[tw] OR "condiment"[tw] OR "condiments"[tw] OR "spice"[tw] OR "spices"[tw] OR "dietary"[tw] OR "diet"[tw] OR "diets"[tw] OR "carbohydrate"[tw] OR "carbohydrates"[tw] OR "grain"[tw] OR "grains"[tw] OR "fiber"[tw] OR "fibers"[tw] OR "prebiotic"[tw] OR "prebiotics"[tw] OR "probiotic"[tw] OR "probiotics"[tw] OR "fruit"[tw] OR "fruits"[tw] OR "seed"[tw] OR "seeds"[tw] OR "nuts"[tw] OR "intake"[tw] OR "vegetable"[tw] OR "vegetables"[tw] OR "flavoring"[tw] OR "flavouring"[tw] OR "Flavonoids"[Mesh] OR "Flavonoids"[tw] OR "Flavonoid"[tw] OR "Anthocyanins"[tw] OR "Anthocyanin"[tw] OR "Catechins"[tw] OR "Catechin"[tw] OR "Flavanones"[tw] OR "Flavanone"[tw] OR "Flavones"[tw] OR "Flavone"[tw] OR "Flavonolignans"[tw] OR "Flavonolignan"[tw] OR "Isoflavones"[tw] OR "Isoflavone"[tw]) AND ("Animal Experimentation"[Mesh] OR "Murinae"[Mesh] OR "murinae"[tw] OR "rat"[tw] OR "rats"[tw] OR "mouse"[tw] OR "mice"[tw] OR "murine"[tw] OR "rodent"[tw] OR "rodents"[tw]) NOT ("humans"[mesh] NOT "murinae"[mesh]). Publication date range was set from the identification of UCP1 as the inner mitochondria component driving the thermogenic process in BAT in 1978 [23], until November 30th, 2019.

RESULTS

A total of 3919 publications were identified in the search (**Fig. 1**). No additional studies meeting the inclusion criteria were identified after adapting the search terms to the Scopus database (data not shown). A total of 143 publications (including 146 different studies) were included after applying the inclusion and exclusion criteria. **Supplemental Table 1** depicts all the plant-based dietary components included, sorted by the number of studies and name of the dietary component, whereas **Supplemental Table 2** shows the study set-ups and the main results of the 146 studies included in this systematic review. Notably, 98 of 146 studies (67%) employed an isolated dietary component or a selected isolated fraction of the plant extracts, whereas the rest (n = 48, 33%) used the whole plant extract. Because of the heterogeneity of the methods and information availability of the included studies, no quality assessment scale system was applied.

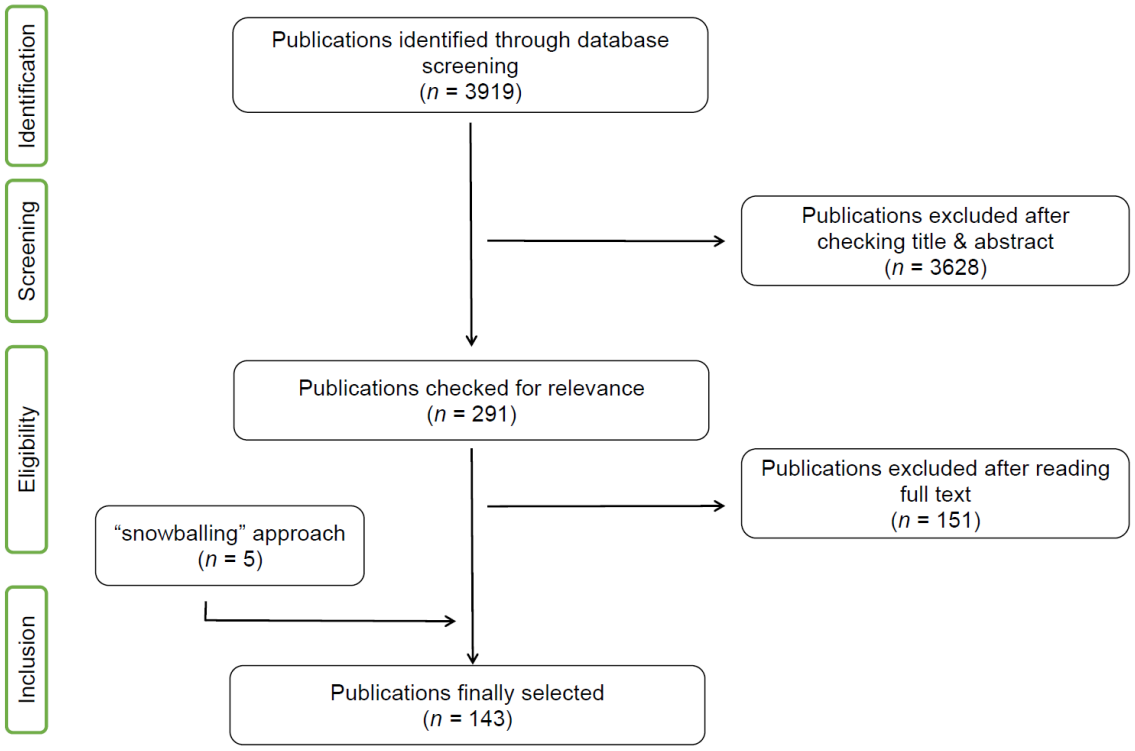

Figure 1. Flowchart of the literature search and study selection process.

General results

For a better comprehension of the results and due to the elevated number of selected studies, we next focused on those plant-based dietary components that were investigated in 6 or more studies. The most studied plant-based dietary components were: i) tea extract catechins (n = 9 studies) [24–32]; ii) resveratrol (n = 8 studies) [33–40]; iii) capsaicin & capsinoids (n = 8 studies) [41–48]; iv) cacao extract flavanols (n = 7 studies) [49–55]; and v) quercetin (n = 6 studies) [56–61], which constitute a subset of n = 38 studies to be considered for the next analysis. (**Fig. 2** and **Table 1**). We repeated these analyses using a less strict threshold ($n \geq 3$) and the studies using monosaccharides/sweeteners, curcumin, leucine, menthol, garlic, and *puerariae* flowers were included (**Fig. 3**). Of the 86 different dietary components included in the 146 studies, 14% were studied twice and 73% were studied only once. Among the subset of 38 selected studies, 96% of the studies that measured UCP1 expression reported a significant activation of BAT [24,25,29–35,37–39,41,42,45,49–55,57], whereas 84% of studies that measured UCP1 expression reported a significant promotion of WAT browning [24,26–28,36,40,42–44,46,56–61]. Some studies found that an upregulation in adenosine monophosphate–activated protein kinase (AMPK) signalling was involved in BAT activation [29,31,38,39,50,52,54,55] and promotion of WAT browning [36,56,57,59]. Accordingly, other studies found that

an upregulation of the Sirtuin 1 (SIRT1) signaling was involved in BAT activation [30,33–35,45] and promotion of WAT browning [44].

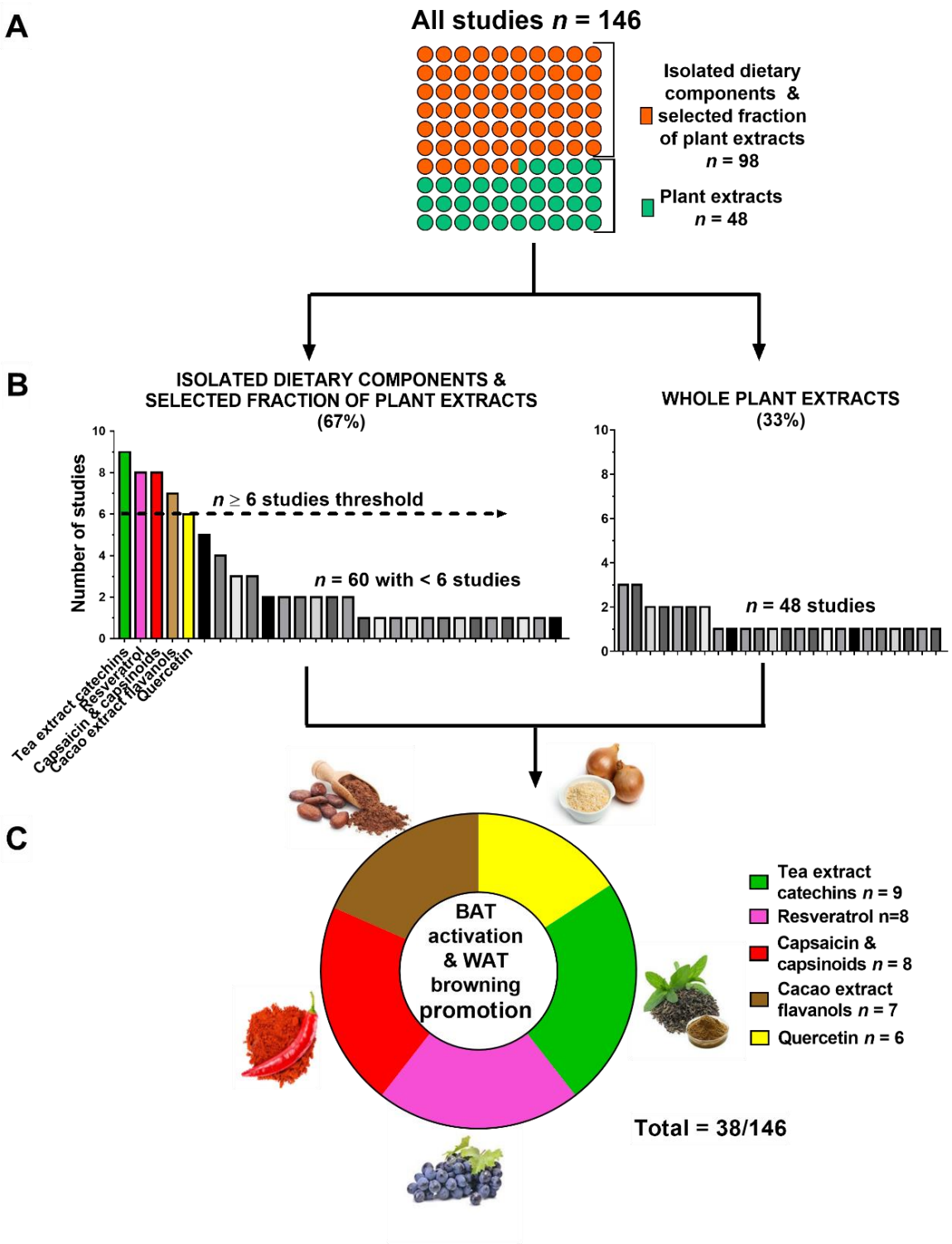

Figure 2. Process to select the most studied plant-based dietary components that activate BAT and/or promote WAT browning in rodents. (A) The number of isolated dietary components & selected fraction of plant extract studies versus plant extracts studies after the initial screening. (B) Histogram depicting isolated the dietary components & selected fractions of plant extracts studies ($n \geq 6$) (left) and plant studies (right). (C) Donut diagram depicting the most studied dietary components. BAT, brown adipose tissue; WAT, white adipose tissue.

Table 1. Summary of the main findings on the effect of plant-based dietary components on BAT activation and WAT browning ($n \geq 6$ studies).

Dietary component	*Studies, n*	Species (sex)	Type	Dose	BAT activation[1]	WAT browning[1]	Ref.
Tea extract Catechins	9	Sprague Dawley rats (male)	Tea catechins extract	100 mg/(kg ·d)	+	+	[24]
		Sprague Dawley rats (male)	Tea catechins extract	0.5/100 g diet	+	?	[25]
		C57BL/6J mice (male)	Decaffeinated green tea catechins	7.7 g/(kg ·d)	?	+	[26]
		Sprague Dawley rats (male)	Tea catechins extract	77.5 mg/kg diet or 155 mg/kg diet	?	+	[27]
		C57BL/6J mice (male)	Green tea leaves extract	0.5% diet	?	+	[28]
		IRC mice (male)	Tea catechins extract	10 mg/kg (single dose)	+	?	[29]
		C57BL/6J mice (male)	Epigallocatechin-3-gallate	2 g/(L · d)	+	?	[30]
		C57BL/6J mice (male)	Epigallocatechin-3-gallate	0.2% wt/wt	+	?	[31]
		C57BL/6J mice (male)	Epigallocatechin-3-gallate	1% wt/wt	+	?	[32]
Resveratrol	8	NR (male mice)	Resveratrol	25 mg/d	+	?	[33]
		Wistar Rat (male)	Resveratrol	30 mg/(kg ·d)	+	?	[34]
		NR (male mice)	Resveratrol	4 kg/(g ·d)	+	?	[35]
		cd1 mice (female)	Resveratrol	0.1% wt/wt	?	+	[36]
		OLETF rats (male)	Resveratrol	10 mg/(kg ·d)	+	?	[38]
		cd1 mice (female)	Resveratrol	0.1% wt/wt	+	?	[39]

		C57BL/6 mice (male)	Resveratrol (R) or oxyresveratrol (OR)	0.5% (R); 0.1% or 0.5% oxyresveratrol (OR)	?	+	[40]
		Wistar Rat (male)	Resveratrol	30 mg/(kg ·d)	+	?	[37]
Capsaicin & capsinoids	8	Std ddY mice (male	Capsiate	10 mg/kg (single dose + 2 wk)	+	~	[41]
		Swiss albino mice (male)	Dihydrocapsiate	2 or 10 mg/(kg ·d)	?	+	[46]
		C57BL/6N mice (male)	Capsinoids	0.3 % wt/wt	~	+	[47]
		C57BL/6J mice (male)	Capsinoids	0.3% wt/wt	?	~	[43]
		C57BL/6J (male)	Capsinoids	0.3% wt/wt	+	~	[48]
		Swiss albino mice (male)	Capsaicin	2 mg/(kg ·d)	+	+	[42]
		B6.129X1 (male)	Capsaicin	0.1% wt/wt	?	+	[44]
		B6.129X1 (male)	Capsaicin	0.003, 0.01, 0.03% wt/wt	+	?	[45]
Cacao extract flavanols	7	C57BL/J mice (male)	Flavan-3-ol fraction	50 mg/(kg ·d)	+	?	[49]
		Wistar rats (male)	Flavan-3-ol fraction	0.2% diet	+	?	[51]
		C57BL/6J mice (male)	(-)-epicatechin	15 mg/(kg ·d)	+	?	[50]
		IRC mice (male)	Flavan-3-ol fraction	10 mg/kg (single dose)	+	?	[52]
		C57BL/6 mice (male)	Cacao liquor procyanidin extract	0.5 or 2% wt/wt	+	?	[55]

		IRC mice (male)	Flavan-3-ol fraction	10 mg/kg (single dose)	+	?	[54]
		IRC mice (male)	B-type procyanidins	10 mg/kg (single dose)	+	?	[53]
Quercetin	6	C57BL/6 mice (male)	Isoquercitrin or quercetin	0.02%, 0.1% and 0.5% wt/wt	?	+	[56]
		C57BL/6 mice (male)	Pentamethylquercetin	0.4% g/g	?	+	[60]
		C57BL/6J mice (male)	Onion peel extract	0.5%	?	+	[59]
		C57BL/6J mice (male)	Quercetin	0.01% wt/wt	~	+	[58]
		C57BL/6J mice (male)	Quercetin	0.05% wt/wt	+	+	[57]
		Sprague–Dawley rats (male)	Quercetin	0.36% and 0.72%	?	+	[61]

[1]BAT activation and WAT browning: (+) UCP1 expression significantly increased, (-) UCP1 expression significantly decreased, (~) UCP1 expression unchanged, (?) UCP1 expression was not measured.

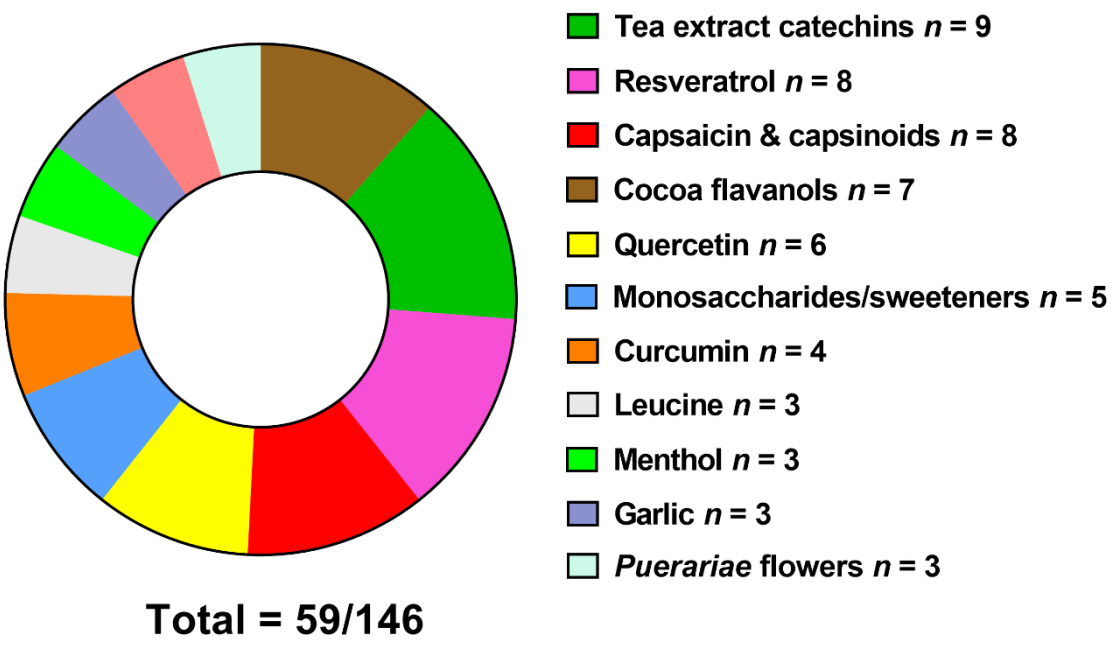

Figure 3. Donut diagram depicting the most studied dietary components after applying a less strict threshold ($n \geq 3$ studies).

Tea catechins, resveratrol, capsaicin, and capsinoids promote BAT activation and WAT browning

Tea extract catechins promoted BAT activation in 6 of 9 studies [24,25,29–32] and increased WAT browning in 4 studies [24,26–28], whereas resveratrol promoted BAT activation in 6 of 8 studies [33–35,37–39], and increased WAT browning in 2 studies [36,40]. Capsaicin and capsinoids promoted BAT activation in 4 of 8 studies [41,42,45,48], while one study showed no BAT activation [43]. Among these 8 studies, 5 reported that capsaicin and capsinoids promoted WAT browning [42–44,46,48], while 3 reported no significant effects on WAT browning [41,43,48].

Cacao extract flavanols activate BAT and quercetin promotes WAT browning

Cacao extract flavanols promoted BAT activation in all studies ($n = 7$) [49–55], while none of them evaluated the effect on WAT browning. Quercetin promoted BAT activation in one of 6 studies [57], while one study reported no BAT activation [58].

DISCUSSION

In this systematic review, we investigated rodent studies evaluating the effects of plant-based dietary components on the activation of BAT and promotion of WAT browning. Tea extract catechins were the most studied plant-based dietary component, followed by resveratrol, capsaicin and capsinoids, cacao flavanols, and quercetin. 67% of the studies used isolated dietary components or a selected fraction of the plant extracts, whereas the remaining 33% used whole plant extracts. AMPK and SIRT1 upregulation were linked to the activation of BAT and promotion of WAT browning. Collectively, these findings support the use of plant-based dietary components as tools to activate BAT and promote WAT browning in rodents and thus potentially combat obesity and cardiometabolic disorders.

Tea consumption from plants of *Camellia sinensis* is one of the world's most consumed beverages [62]. The thermogenic response to tea extract catechins seems to be driven by the transient receptor potential vanilloid subfamily member 1 (TRPV1) and TRP ankyrin 1 (TRPA1) channels expressed in the gut [63], and brown and white adipocytes' membranes [64]. Interestingly, the most abundant and bioactive tea catechin, epi-gallocatechin gallate (EGCG), and its autoxidation products can activate TRPV1 and TRPA1 in intestinal enteroendocrine cells at equivalent doses to those expected in the gut after tea catechins ingestion [65,66]. Therefore, it is likely that tea catechins could activate BAT via TRP channels located in the sensory neurons of the gut via a gut-sympathetic nervous system (SNS)-BAT axis (68). BAT activation by tea catechins will be ultimately driven via sympathetic activation of β-ARs on brown adipocytes and by the inhibition of catechol-O-methyl transferase (COMT) by tea catechins, a catecholamine-degrading enzyme [67,68]. However, COMT activity is not inhibited by high doses of EGCG in humans, indicating a negligible role of COMT in the catechin effects in vivo [69,70]. This is explained because of the much lower circulating levels of catechins after single ingestion (~0.1μM at maximum) [71] compared with the half-maximal inhibitory concentration for the COMT activity (~14μM) [72]. Notably, green tea leaves extracts are also rich in caffeine, a phosphodiesterase inhibitor [73]. phosphodiesterase enzyme that degrades cAMP and that could enhance the protein kinase A (PKA) thermogenic pathway, as PKA is positively allosterically modulated by cAMP [74]. Nonetheless, further studies are needed to confirm a link between tea extract catechins and the activation of the gut-SNS-BAT axis.

Capsaicinoids is a term that refers to a sub-group of secondary metabolites of the genus *capsicum* plant, known for being pungent. The most important capsaicinoid is capsaicin, being responsible for the pungent effects of chili peppers through the activation of TRPV1 channels in the gut [75]. Capsinoids,

which include capsiate, dihydro-capsiate, and nor-dihydro-capsiate, activate TRPV1 and TRPA1 channels being significantly less pungent than capsaicin [76]. We observed that capsaicin and capsinoids activated BAT and promoted WAT browning in rodents, probably via SNS adrenal catecholamine secretion [77]. Congruently, the intragastric administration of capsinoids promoted BAT activation via TRPV1 agonism and sympathetic activation [77]. However, BAT activation after capsinoids treatment was abolished after vagal afferent denervation [78] or in UCP1-KO mice [48]. These results strengthen the idea that capsaicin and capsinoids effects rely on a gut-SNS-BAT axis.

Resveratrol is one of the most well-known polyphenols with antioxidant properties. It can mainly be found in grapes' skin and seeds, but also in berries and nuts. Resveratrol activates BAT [33–35,37–39] and promotes WAT browning in mice and rats [36,40], but the gut-SNS-BAT connection has not yet been demonstrated. Instead, it seems that the thermogenic properties of resveratrol are directly mediated at the intracellular level, by an up-regulation of the thermogenic pathways and related-makers such as FNDC5 (type I membrane protein) and SIRT1 [79]. Nevertheless, further investigation is warranted to confirm which mechanisms are driving BAT activation after resveratrol ingestion.

Cacao beans are rich in flavonoids that constitute up to 10% of the dry weight of the bean [80]. Cacao extract flavanols promote BAT activation, but the extent that cacao flavanols can promote WAT browning remains unexplored. BAT activation by cacao extract flavanols is driven by an increase in catecholamine secretion and the consequent activation of the β-ARs on brown adipocytes [52–54]. Furthermore, it is important to consider that cacao extract flavanols can also be a source of theobromine and caffeine, substances that can boost sympathetic response [81] and thereby affect BAT activation. Even though BAT activation seems to be mediated by adrenergic activation, further studies are needed to confirm this hypothesis.

Quercetin is the most abundant flavonoid in onions, yet it can also be found in other vegetables and fruits. Similar to cacao flavanols, quercetin seems to drive its thermogenic activation through sympathetic stimulation [57]. The promotion of WAT browning by quercetin could be explained by a higher sensibilization of white adipocytes to catecholamines, as quercetin upregulates β3-AR in WAT [57]. However, the thermogenic mechanisms explaining the promotion of WAT browning by quercetin remain to be elucidated.

Lastly, we have analyzed in-depth those studies included after applying the sensitivity threshold of $n \geq 3$, *i.e.*, monosaccharides/sweeteners, curcumin, leucine, menthol, garlic, and *puerariae* flowers. However, the current evidence is

not strong enough to support these plant-based dietary components as BAT activators and promoters of WAT browning and further investigation is warranted to confirm these findings.

AMPK is considered one of the major controllers of the cellular response to energetic stress and mitochondrial homeostasis [82], playing a significant role in the development and metabolism of brown and beige adipocytes [83]. Previous AMPK-null mice studies have shown that AMPK is necessary for cold-induced and β-adrenergic BAT activation and WAT browning [84]. Congruently, the specific pharmacological activation (A-769662) of AMPK has been shown to promote WAT browning [85]. SIRT1, which also has fuel-sensing properties similar to AMPK, is important for the activation of BAT and promotion of WAT browning [86]. A whole-body SIRT1 heterozygous knockout ($SIRT1^{+/-}$) mouse model study showed a decrease in BAT activity, higher adiposity, and insulin resistance [86], suggesting that SIRT1 activation is needed for normal BAT function. Several of the studies reviewed herein reported that AMPK and SIRT1 pathways were upregulated by dietary components in both BAT and WAT.

BAT has an important endocrine role orchestrated by the release of batokines, with an impact on metabolism both at local and systemic levels [12,13]. Therefore, it is not surprising that many the of studies included in this systematic review also reported significant improvements in glucose and lipid metabolism along with BAT activation/recruitment and/or WAT browning. Collectively, these findings suggest that the potential clinical relevance of dietary components goes beyond thermogenic effects, as BAT activation and WAT browning might be driving the additional metabolic improvements potentially through BAT-mediated endocrine mechanisms. Future studies should address the connection between the secretory role of BAT and the metabolic improvements elicited by dietary components.

Limitations of the systematic review

Selected dietary components: It is important to highlight that although we considered a reasonably wide spectrum of dietary components, the search strategy focused on a select group of compounds (see **Methods** section). Thus, certain groups of compounds, such as carotenoids were not explicitly included.

Species included in the search: The present systematic review is focused on studies conducted exclusively in rodent models (mice and rats). Thereby, these findings could not be transferable to other species.

In the light of the aforementioned limitations, we contend that future systematic reviews on this topic should focus on specific and well-defined groups

of dietary components. This would strengthen the scope of the studies by allowing them to employ more specific search strategies and to include a wider spectrum of animal models (*i.e.,* non-rodent).

Limitations and possible bias of the included studies

Heterogenous composition of plant-based dietary component extracts: For instance, There were differences in the composition of those studies using tea extract catechins [24–29] and cacao extract flavanols [50,53,55]. Thus, a comparison of the results between these studies should be done carefully. Future studies should standardize the composition of plant-based dietary components to enable a critical comparison of the results.

***Dose*:** Dose variations of the plant-based dietary components used in the studies also complicate the comparison of results between studies.

Authorship: It is of note that 5 of 7 studies that used cacao extract flavanols were conducted by the same lab [49,51–54]. The Ajinomoto Company was involved in 3 of 4 studies using capsinoids [43,47,48], whereas 2 of 3 capsaicin studies were conducted by members of the same lab [44,45]. 2 of 8 resveratrol studies were conducted by a USA-China collaboration [36,39], while a Spanish group conducted 2 of 8 studies [34,37]. Therefore, these findings should be replicated by independent labs.

Analysis of thermogenic pathways: Given that AMPK and SIRT1 assessments were dependent on the arbitrary selection of this outcome by authors, no firm conclusions could be drawn from this data until further studies using unbiased approaches (e.g., RNA sequencing and proteomics) confirm these hypotheses.

Assessment of BAT activity: The studies included lack of evaluation of actual BAT activity through either 18F-FDG-PET/CT scans (the current gold standard [87]), direct interscapular BAT (IBAT) temperature measurements, or infrared thermography assessments. Notably, it is important to consider that many of the studies only measured UCP1 at the gene expression level, which cannot be considered a proxy of thermogenesis. While mRNA/protein ratio is thought to be constant [88], it could vary depending on specific tissues and genes. Thus, future studies should include UCP1 protein assessments in their analysis.

BAT activation and BAT recruitment: Most of the studies evaluated the effects of plant-based dietary components over a period of time by measuring UCP1 mRNA or protein levels in BAT, which reflects BAT recruitment. Even though BAT recruitment is likely a consequence of repeated BAT activation, only a capsinoids study (42) and two cacao flavanol extract studies (53,55) showed an acute activation of pre-existing BAT. Thus, since for BAT to display anti-obesity

effects, it also needs to be active, future studies should prove an actual BAT activation (and not only recruitment) after chronic plant-based dietary components interventions.

Safety of plant-based dietary components: It has been shown that certain catechins at concentrations may be responsible for the hepatoxic effects of green tea extract [70], or β-carotene supplementation could increase the risk of lung cancer in smokers [89]. Thus, future studies investigating the safety of supplementations with plant-based dietary components are needed.

Translational research: future lines

Only two studies have evaluated the effect of oral tea extract catechins on human BAT activity [90,91], showing that tea extract catechins increase cold-induced thermogenesis, resting metabolic rate, and BAT density in BAT-positive individuals. Sun et al. reported a significant increase in BAT glucose uptake after capsinoids ingestion [92]. In light of the present results, resveratrol, cacao extract flavonols and quercetin could be potential activators of human BAT, although their properties have never been tested as BAT activators in humans. Furthermore, whether plant-based dietary components promote WAT browning in humans remains unexplored. Experimental procedures, robust study designs, and use of the gold standard techniques for assessing BAT activity, and WAT browning (biopsies; transcriptomics, and proteomics) must be used in future studies.

CONCLUSION

To date, the most studied plant-based dietary components for activating BAT and promoting WAT browning in mice and rats are tea extract catechins, resveratrol, capsaicin and capsinoids, cacao extract flavanols and quercetin. The findings of the present systematic review support the use of plant-based dietary components as tools to activate BAT and promote WAT browning in rodents and thus potentially combat obesity and cardiometabolic disorders. It seems that a part of these effects could be dependent on the upregulation of AMPK and SIRT1 signaling, yet further studies are needed to confirm the mechanisms driving these results. Studies in humans are warranted to understand the impact of plant-based dietary components on BAT metabolism and WAT browning.

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SUPPLEMENTARY MATERIAL (STUDY IV)

SUPPLEMENTARY MATERIAL

Supplemental Table 1. List of plant-based dietary components (isolated dietary components, selected isolated fraction of the plant extracts, and whole plant extracts), sorted by number of studies.

Dietary component	Number of studies	Reference
Tea extract catechins	9	1–9
Capsaicin and capsinoids	8	10–16
Resveratrol	8	17–24
Cacao extract flavanols	7	25–31
Quercetin	6	32–37
Monosaccharides and sweeteners	5	38–42
Curcumin	4	43–46
Garlic	3	47–49
Leucine	3	50–52
Menthol	3	53–55
Puerariae flower extract	3	56–58
B-laphacone	2	59,60
Bofutsushosan	2	61,62
Calcium	2	63,64
Cinnamaldehyde	2	65,66
Fucoxanthin	2	67,68
Guarana	2	69,70
Luteolin	2	71,72
Oleuropein aglycone	2	73,74
Olive oil	2	75,76
Platycodon grandiflorus Root Ethanol Extract	2	77,78
Raspberry	2	79,80
Vitamin A	2	81,82

Albiflorin	1	[83]
Allicin	1	[84]
Artepillin C	1	[85]
***Atractylodes macrocephala* Koidzumi**	1	[86]
Barley Extracts with *Lactobacillus Plantarum dy-1*	1	[87]
Berberine	1	[88]
Bilberry (fiber)	1	[89]
Bitter melon seed oil	1	[90]
Black Soybean Seed Coat Extract	1	[91]
Blueberry Extract	1	[92]
Borage oil	1	[93]
Butein	1	[94]
Capsaicin + Hesperidin	1	[95]
Caulis Spatholobi	1	[96]
Chrysanthemum Leaf Ethanol Extract	1	[72]
Cinnamon	1	[97]
Citrus reticulata	1	[98]
Cordycepin	1	[99]
Curcumin + Artepillin C	1	[100]
Ellagic acid	1	[101]
Formononetin	1	[102]
Genistein	1	[103]
Ginger	1	[104]
Glucoraphanin	1	[105]
Grape pomace extract	1	[106]
Grape seed proanthocyanidin extract	1	[107]
Gypenosides	1	[108]
Histidine	1	[109]

Inorganic Nitrate	1	110
Jinlida	1	111
Kaempferia parviflora extract	1	112
Limonoid 7-Deacetoxy-7-oxogedunin (Carapa guianensis)	1	113
Lyophilized Maqui *(Aristotelia chilensis)*	1	114
Matured hop bittering components	1	115
Melinjo *(Gnetum gnemon L.)* seed extract	1	116
Momordica charantia	1	117
Mulberry leaves	1	118
Myrciaria dubia	1	119
Nigella sativa extract	1	120
Nitzschia laevis extract	1	121
Octacosanol and policosanol	1	122
Omija fruit extract	1	123
Oolang , black and pu-erh tea extract	1	124
Out of season orange	1	125
Panax ginseng	1	126
Phaeodactylum tricornutum extract	1	127
Phosphate	1	128
Phytol	1	129
Plantago asiatica extract	1	130
***Psoralea corylifolia L.* (Prenylated flavonoid-standardized extract)**	1	131
Quercetin + resveratrol	1	132
Rojal Jelly and Bee Larva Powder	1	133
Rose hip	1	134
Rubi Fructus *(Rubus coreanus)*	1	135
Rutin	1	136
Sesaminol diglucoside	1	137

Soy Isoflavones	1	138
Thymoquinone	1	120
Undaria pinnatifida	1	68
Vanillic Acid	1	139
Wheat gluten	1	140
α/γ-Tocoferol	1	141
α-Monoglucosyl Hesperidin and Hesperidin	1	142

The black line in the middle shows the threshold of n≥6, and the dash line shows the threshold (n≥3) used for sensitivity analyses.

Supplemental table 2. Detailed information about the methodology and results published in the manuscripts included in this systematic review (alphabetically sorted).

Dietary component	Dose	Species (sex)	Age, wk	Study duration, wk	Housing Temperature,[1] °C	12 h light:dark cycle	Humidity, %	*Ad libitum* food and water	Diet	Sample size	BAT activation	WAT browning	Ref.
Albiflorin	5 mg/(kg · d)	C57BL/6J mice (male)	4	6	NR	NR	NR	NR	HFD	*n* = 5	BAT: *Ucp1, Pgc1a, Cidea* (qRT-PCR); UCP1, PGC1a (inmunoblot)	NR	[83]
Allicin	1 mg/kg (every 2 days)	C57BL/6J mice (male)	6	8	NR	Y	NR	Y	HFD	*n* = 5	NR	iWAT: *Ucp1, Prdm16* (qRT-PCR); UCP1, KLF15 (inmunoblot); UCP1, KLF15 (immunostaining)	[84]
Artepillin C	5 mg/(kg · d) and 10 mg/(kg · d)	C57BL/6J mice (male)	7-9	4	23 ± 3	Y	NR	Y	STD	NR	BAT: No effect on UCP1 nor COXIV (inmunoblot)	iWAT: UCP1, PRDM16 (immunostaining)	[85]
***Atractylodes macrocephala* Koidzumi**	100 mg/(kg · d) and 300 mg/(kg · d)	C5BL/6 (male)	5	16	22-23	Y	NR	Y	HFD	*n* = 5	BAT: UCP1 (300 mg/kg vs. Control, inmunoblot); PCG1a (100 and 300mg/kg vs. Control, inmunoblot)	NR	[86]

Barley Extracts with Lactobacillus Plantarum dy-1	800 mg/kg	Sprague-Dawley Rats (male)	8	10	22 ± 2	Y	40%-60	Y	HFD	*n* = 8	BAT: *Ucp1, Pgc1a, Cox* (rt-qPCR); UCP1 (inmunoblot)	eWAT: *Ucp1, Pgc1a, Cox* (qRT-PCR); no UCP1 differences (inmunoblot)	(144)
Berberine	100 mg/(kg · d)	C57BL/6J mice (male)	4	16	NR	NR	NR	NR	NR	*n* = 5	BAT: UCP1 (inmunotoblot)	sWAT: UCP1 (inmunotoblot)	[88]
Bilberry (fiber)	7% (dry weigh basis)	Sprague–Dawley rats (male)	4	8	23 ± 1	Y	60 ± 5	Y	LFD and HFD	*n* = 8	BAT: *Ucp1* (RT-qPCR)	sWAT: No effect on *Ucp1* (RT-qPCR)	[89]
Bitter melon seed oil	5, 10 or 15% diet	C57BL/6J mice (male)	9	11	NR	NR	NR	NR	HFD	*n* = 6	NR	rWAT: *Ucp1, Tmem26,Tbx1, Adbr3, Ppara, Ppargc1a* (qRT-PCR); UCP1 (immunostaining and inmunoblot)	[90]
Black Soybean Seed Coat Extract	0.2%, 1% and 2% wt/wt	C57BL/6J mice (male)	5	14	22 ± 3	Y	NR	Y	STD and HFD	*n* = 6	BAT: *Ucp1* (2% HFD vs. 0% HFD; qRT-PCR); UCP1 (2% HFD vs. 0% HFD; inmunoblot)	sWAT:no effects on *Ucp1* (qRT-PCR); UCP1 (2% HFD vs. 0%, inmunoblot)	[91]
Black Soybean Seed Coat Extract	22g extract/kg diet												

B-laphacone	20 mg/(kg · d) and 40 mg/(kg · d)	C57BL/6 mice (male)	4	11	21-25	Y	50-60	Y	HFD	NR	BAT: *Ucp1, Ppara, Prdm16* (low dose, qRT-PCR); *Ucp1, Pgc1a, Cidea, Ppara, Prdm16* (high dose, qRT-PCR)	sWAT: *Ucp1, Prdm16, Pgc1a, Cidea, Elovl3, Cox8b* (qRT-PCR, high dose); epWAT: *Ucp1, Prdm16, Pgc1a, Cidea, Elovl3* (qRT-PCR, high dose); sWAT: *Ucp1* (qRT-PCR, low dose); eWAT: *Prdm16, Pgc1a, Cidea* (qRT-PCR, low dose)	[59]
B-laphacone	5 mg/(kg · d)	C57BL/6J mice (male)	8	10	NR	NR	NR	NR	HFD	n = 5	BAT: *Ucp1, Pgc1a, Cidea, Sirt3* (qRT-PCR)	sWAT: UCP1 (immunostaining)	[60]
Blueberry Extract	5 g/L	C57BL/6 mice (male)	4	14	22 ± 2	Y	50 ± 15	Y	HFD	n = 9-12	BAT; *Ucp1, Prdm16, Pgc1a* (qRT-PCR); UCP1, PRDM16, Pgc1α (inmunoblot)	iWAT: *Ucp1, Prdm16, Pgc1a* (qRT-PCR)	[92]
Bofutsushosan	7.2 g/(kg · d)	IRC mice (female)	20	7	22-25	Y	40-60	Y	HFD	n = 6	BAT: *Ucp1, Pgc1a* (qRT-PCR)	NR	[61]

Bofutsushosan	1% wt/wt	C57BL/6 mice (male)	6	4	24 ± 2	Y	45 ± 5	Y	HFD	*n* = 5-6	BAT: no effect on *Ucp1* (qRT-PCR)	eWAT: *Ucp1* (qRT-PCR)	[62]
Borage oil	borage oil (g-linolenic acid 25%) or borage oil (g-linolenic acid 47%)	Sprague–Dawley rats (male)	6	2	20–22	Y	55-60	Y	HFD	*n* = 7	BAT: *Ucp1* (qRT-PCR)	NR	[93]
Butein	5 mg/(kg · d) and 15 mg/(kg · d)	C57BL/6N mice (male)	8	6	NR	Y	NR	NR	HFD	*n* = 7	BAT (low dose): *Ucp1* (qRT-PCR); BAT (high dose): *Ucp1, Prdm4* (qRT-PCR)	iWAT (low dose): no effect on *Ucp1, Prdm4* (qRT-PCR); iWAT (high dose): *Ucp1, Prdm4* (qRT-PCR); eWAT (low dose): *Prdm4* but not *Ucp1* (qRT-PCR); eWAT (high dose): *Ucp1, Prdm4* (qRT-PCR) - UCP1, PRDM4 (inmunoblot)	[94]

Cacao liquor procyanidin extract	0.5 or 2% wt/wt	C57BL/6 mice (male)	5	13	23 ± 2	Y	NR	Y	STD and HFD	*n* = 6	BAT: *Ucp1* (0,5 and 2% HFD vs. 0% HFD, qRT-PCR); *Pgc1a* (2% vs. 0,5 and 0% STD; 0,5 an0d 2% v 0% HFD)	NR	[31]
Calcium	12 g/(kg · d)	C57BL/6 mice (male)	5	10	22	Y	NR	Y	HFD	*n* = 6	BAT: no effect on *Ucp1* (qRT-PCR) nor UCP1 (inmunoblot)	NR	[63]
Calcium	0.6% wt/wt	C57BL/6J mice (female)	8	20	NR	Y	NR	Y	HFD	*n* = 12	BAT: *Ucp1, Prdm16, Pgc1a, Cidea, Elovl3* (qRT-PCR); UCP1 and PGC1A (inmunoblot)	sWAT: UCP1, PGC1A (inmunoblot)	[64]
Capsaicin	2 mg/(kg · d)	Swiss albino mice (male)	6-7	12	22 ± 1	Y	55 ± 5	Y	HFD	*n* = 8	BAT: *Ucp1, Cidea, Ppara, Pgc1a, Sirt1* (qRT-PCR)	sWAT: *Ucp1, Cidea, Ppara, Pgc1a, Sirt1* (qRT-PCR)	[11]
Capsaicin	0.1% (wt · wt)	B6.129X1 (male)	6	26	23	NR	NR	Y	STD or HFD	*n* = 40	NR	sWAT:*Ucp1, Ppara, Pparg, Prdm16, Sirt1, Pgc1a, Bmp8b* (qRT-PCR); UCP1, PGC1A, PPARg, PRDM16, BMP8b (inmunoblot); eWAT: Ucp1, *Ppara, Pparg, Prdm16, Sirt1,*	[13]

												Pgc1a (qRT-PCR); downregulation of UCP1, PPARA, PPARG (inmunoblot)	
Capsaicin	0.003, 0.01 and 0.03% wt/wt	B6.129X1 (male)	6	32	22.8 ± 2.0	Y	45-50	Y	HFD	*n* = 5	BAT: *Ucp1, Bmp8b, Prdm16, Pgcc1a, Sirt1, Ppara, Pparg* (qRT-PCR); UCP1, BMP8B, PRDM16, PGC1A, PPARA, PPARG (inmunoblot)	NR	14
Capsaicin + Hesperidin	Hesperidin (HESP: 100 mg/(kg · d)); Capsaicin (CAP: 4 mg/(kg · d)); HESP+CAP (100 mg/(kg · d) + 4mg/(kg · d)	Wistar rats (male)	13	8	22	Y	NR	Y	WD	*n* = 8	BAT (HESP): UCP1 (inmunoblot)	rWAT (HESP and CAP): UCP1 and CIDEA (immunostaining); iWAT (CAP): No effect on *Ucp1* nor *Pgc1a*, but yes in *Prdm16* and *Cidea* (qRT-PCR); no effect on browning genes with HESP or HESP + CAP; rWAT: no effect on *Ucp1* (all groups, qRT-	95

												PCR); rWAT (CAP vs HESP): UCP1 (inmunoblot); rWAT (all groups): no changes for CIDEA (inmunoblot); iWAT (CAP and HESP + CAP vs HESP): UCP1 (inmunoblot); iWAT (CAS and HESP): CIDEA (inmunoblot)	
Capsiate	10 mg/(kg · d)	Std ddY mice (male)	6	2	22 ± 0.5	Y	50	NR	Stock diet (MF type)	*n* = 9-10	BAT: *Ucp1* (qRT-PCR); UCP1 (inmunoblot)	eWAT: no effect on *Ucp1* (qRT-PCR)	[10]
Capsinoids	0.3% wt/wt	C57BL/6J mice (male)	9	8	25 ± 1.0	lights on from 1600 to 0400	NR	Y	HFD	*n* = 7-8	NR	sWAT: no effect on *Ucp1* (qRT-PCR)	[12]
Capsinoids	0.3 % wt/wt	C57BL/6N mice (male)	8	8	17 ± 1	Y	NR	Y	HFD	*n* = 6	BAT: no differences for *Ucp1, Pgc1a, Cidea, Cox8b* (qRT-PCR)	iWAT: *Ucp1, Pgc1a, Cidea, Cox8* (qRT-pPCR)	[12]

Capsinoids	0.3 % wt/wt	C57BL/6J (male)	9	12	23 °	Y	NR	Y	HFD	*n* = 12	BAT: UCP1 (inmunoblot);	pWAT: no effect on UCP1 (inmunoblot);	[16]
Caulis Spatholobi	0.25%, 0.5%, 1% wt/wt	C57BL/6J mice (male)	5	13	22 ± 2	13 h light:dark cycle	55 ± 10	Y	HFD	*n* = 8	BAT: *Ucp1, Pgc1a, Prdm16* (qRT-PCR); UCP1 (inmunoblot)	sWAT: *Ucp1, Pgc1a, Prdm16* (qRT-PCR); UCP1 (inmunoblot)	[96]
Chrysanthemum Leaf Ethanol Extract	1.5% wt/wt	C57BL/6 mice (male)	4	16	24	Y	NR	Y	HFD	*n* = 10	BAT: UCP1 (immunostaining)	eWAT:*Ucp1, Cox8b, Cidea, Adrb3, Ppara, Pgc1a and Ppargc1b* (qRT-PCR)	[72]
Cinnamaldehyde	0,1%, 0,5% and 1%	C57BL/6J mice (male)	5	4	23 ± 1	Y	55 ± 5	Y	HFSD	*n* = 6	BAT: UCP1 (0,5 and 1% inmunoblot)	NR	[66]
Cinnamaldehyde	40 mg/(kg · d)	C57BL/6J mice (male)	12	8	23 ± 2	Y	55 ± 10	Y	HFD	*n* = 8	BAT: PRDM16 and PGC1A (inmunoblot). No effect on UCP1	eWAT: UCP1 (inmunoblot); *Prdm16* (qRT-PCR)	[65]
Cinnamon	500 mg/(kg · d)	C57BL/6 (male)	13	2	NR	Y	NR	Y	HFD	*n* = 3	NR	sWAT: *Ucp1 and Prdm16* (qRT-PCR, no changes for Cidea); UCP1 (immunostaining)	[97]

Citrus reticulata	1% wt/wt	C57BL/6 mice (male)	6	11	25 ± 1	Y	NR	Y	STD and HFD	*n* = 8	NR	iWAT: *Ucp1* (no *Cidea, Tmem26, cd137*, qRT-PCR), UCP1 (immunostaining), UCP1, but no PGC1A (inmunoblot),	98
Cordycepin	40mg/(kg · d)	C57BL/6 mice (male)	NR	3	NR	NR	NR	NR	HFD	*n* = 7	NR	iWAT: *Ucp1, Cidea, Pgc1a* (qRT-PCR); UCP1, PGC1A (inmunoblot); no differences in eWAT	99
Curcumin	1.5 or 4.5 mg/(kg · d) (highly dispersible and bioavailable, HC) and 4.5 mg/(kg · d) (native, N)	C57BL/6 mice (male)	5	4	23 ± 3	Y	NR	Y	HFD	*n* = 10	BAT: no effect on UCP1 nor COXIV	iWAT: UCP1 (4,5 HC, immunostaining in); no effect on eWAT	46
Curcumin	50 or 100 mg/(kg · d)	C57BL/6 mice (male)	NR	NR	22 ± 2	Y	55 ± 5	Y	HFD	*n* = 6-7	NR	iWAT: *Ucp1, Dio2, Prdm16, Pgc1a, Cidea, Evolv3* (qRT-PCR); UCP1 (inmunoblot) both doses; no effect on eWAT	43

Curcumin	40 or 80 mg/(kg · d)	C57BL/6 mice (male)	6	12	NR	Y	NR	NR	HFD	*n* = 8	BAT: *Ucp1* (qRT-PCR)	WAT: *Ucp1* (qRT-PCR)	[44]
Curcumin	1%	C57BL/6 mice (male)	NR	6 or 17	22	Y	NR	Y	HFD	*n* = 8	BAT: UCP1 (inmunoblot)	iWAT: no effect on UCP1 (inmunoblot)	[45]
Curcumin + Artepillin C	Cu 1.5 mg/kg + ArtC 5 mg/kg	C57BL/6 mice (male)	5	4	23 ± 3	14 h light:dark cycle	NR	Y	HFD	*n* = 10	BAT: no differences for UCP1 (inmunoblot nor immunostaining)	iWAT: UCP1 (inmunoblot and immunostaining)	[100]
Dihydrocapsiate	2 or 10 mg/kg	Swiss albino mice (male)	5-6	12	22 ± 2	Y	55 ± 5	Y	HFD	*n* = 8	NR	vWAT (10 mg/kg): *Ucp1, Pgc1a, Cox7a,and Tmem26* (qRT-PCR); sWAT (10 mg/kg): *Pgc1a, Tmem26 and cd137* (qRT-PCR); sWAT: *Cd137* (2 mg/kg, qRT-PCR)	[15]
Ellagic acid	10 mg/((kg · d) or 30 mg/(kg · d)	SD rats (male)	6	24	21 ± 2	Y	50 ± 10	Free acccess	HFD	*n* = 8	BAT: *Ucp1, Pgc1a* (qRT-PCR)	iWAT: *Ucp1, Prdm16, Cidea, Pgc1a* (qRT-PCR); UCP1 (immunostaining)	[101]

Epigallocatechin-3-gallate (EGCG)	2 g/L	C57BL/6J mice (male)	13	16	22 ± 2	Y	50 ± 15	Y	HFSD	*n* = 30	BAT: UCP1 (immunostaining)	NR	[7]
Epigallocatechin-3-gallate (EGCG)	1% wt/wt	C57BL/6J mice (male)	5	4	NR	Y	NR	Y	STD and HFD	*n* = 8	BAT: *Ucp1, Pgc1a, Prdm16* (qRT-PCR)	NR	[9]
Epigallocatechin-3-gallate (EGCG)	0.2% wt/wt	C57BL/6J mice (male)	13	8	22 ± 1	Y	NR	Y	HFD	*n* = 6	BAT: *Ucp1, Prdm16, Pgc1a, Cpt1b*	NR	[8]
Flavan-3-ols (cacao extract)	50 mg/(kg · d)	C57BL/J mice (male)	7	2	23 - 25	Y	NR	NR	STD	*n* = 6	BAT: UCP1 (inmunoblot)	NR	[25]
Flavan-3-ols (cacao extract)	0.2%	Wistar rats (male)	8	4	23 - 25	Y	NR	NR	HFD	*n* = 8	BAT: UCP1 (HFD 0,2%inmunoblot)	NR	[27]
Flavan-3-ols (cacao extract)	10 mg/kg	IRC mice (male)	8-10	acute effect	23–25	Y	NR	Y	NR	*n* = 8	BAT: *Ucp1, Pgc1a* (qRT-PCR)	NR	[30]
Flavan-3-ols (cacao extract)	10 mg/kg	IRC mice (male)	8-10	acute effect	23–25	Y	NR	Y	NR	*n* = 8	BAT: *Ucp1* (qRT-PCR)	NR	[29]

Flavan-3-ols (cacao extract)	10 mg/kg	IRC mice (male)	NR	Acute effect	23 - 25	Y	NR	Y	STD	*n* = 8	BAT: *Ucp1, Pgc1a* (qRT-PCR)	NR	[28]
Flavanol, (-)-epicatechin	15 mg/(kg · d)	C57BL/6J mice (male)	35	2	NR	NR	NR	Y	HFD	*n* = 5	BAT: UCP1, DIO2, PRDM16 (inmunoblot)	NR	[26]
Formononetin	01, 1, 10 mg/kg	C57BL/ mice (male)	NR	12	23 - 24	Y	50-60	Y	HFD	*n* = 10	NR	WAT: *Ucp1, Evolv3, Dio2* (high doses, qRT-PCR)	[102]
Fucoxanthin	0,.05 and 0.2% wt/wt	C57BL/6N mice (male)	5	6	22 ± 2	Y	NR	Y	HFD	*n* = 10	BAT: *Ucp1* (0,2%, qRT-PCR). No effect on Ucp1 with 0,05%	eWAT:*Ucp1* (0,05%, qRT-PCR); no effect in other WATs	[67]
Fucoxanthin	1 mg/kg	Wistar rats (male)	7	8	24 ± 2	Y	40-60	Y	HFD	*n* = 6	NR	rWAT: *Ucp1* (qRT-PCR)	[68]
Garlic	2 and 5% wt/wt	C57BL/6J (male)	8	7	22 ± 1	Y	NR	Y	HFD	*n* = 6	BAT:*Ucp1* (qRT-PCR)	sWAT:*Ucp1* (qRT-PCR)	[47]
Garlic	8g/kg diet	Sprague-Dawley Rats (male)	4	4	22 - 24	Y	50	Y	Shortening and HFD	*n* = 6-7	BAT: UCP1 (both diets, inmunoblot)	NR	[48]

Garlic (aged black)	250 mg/(kg · d)	Sprague-Dawley Rats (male)	12	5	22 - 24	Y	50-60	Y	HFD + 5% sucrose (water added)	*n* = 12	BAT: *Ucp1* (qRT-PCR)	NR	[49]
Genistein	0.25 g/(kg · d)	C57BL/6J (female)	5	8	20 - 24°	Y	NR	Y	HFD	*n* = 8	NR	sWAT:*Ucp1, Cidea* (qRT-PCR), no effect on *Pgc1a, Ppara, and Pparg* (qRT-PCR)	[103]
Ginger	500 mg/(kg · d)	C57BL/6J (male)	5	16	25 ± 2	Y	NR	Y	STD and HFD	*n* = 8	BAT: UCP1, PRDM16 and CIDEA (qRT-PCR and inmunoblot); UCP1 (inmunofluorescence)	*Tmem26 and Cited1* (qRT-PCR and inmunoblot)	[104]
Glucoraphanin	0.3% wt/wt	C57BL/6JSlc mice (male)	8	14	24–26	Y	NR	Y	STD and HFD	*n* = 8	BAT: No effect on *Ucp1, Pgc1a, Dio2* (NC nor HFD; qRT-PCR)	eWAT and iWAT: *Ucp1* (HFD, qRT-PCR)	[105]
Grape pomace extract	300 mg/(kg · d)	WKY rats (male)	9	10	23 ± 1°	Y	NR	NR	HFD	*n* = 6	NR	eWAT: no effect on *Ucp1, Pgc1a, Pparg, Prdm16* (qRT-PCR); UCP1 (inmunoblot), no effect on PGC1A, PPARg, PRDM16 (inmunoblot);	[106]

Grape seed proanthocyanidin extract	250 mg/kg	Wistar Rats (male)	9	acute effect	22	Y	NR	Y	STD	*n* = 6	BAT: No effect on *Ucp1*, but upregulation of *Pgc1a, Sirt1* (qRT-PCR)	NR	[107]
Guarana	0.5% wt/wt	Wistar rats (male)	10	18	21	Y	NR	Y	STD or WTD	*n* = 12	BAT: *Ucp1* (WTD, qRT-PCR)	NR	[69]
Guarana (Paullinia cupana)	1 g/(kg · d)	C57BL/6J (male)	4	8	22 ± 2	Y	NR	NR	HFD	*n* = 6	BAT: *Ucp1* (qRT-PCR); UCP1, PGC1A (inmunoblot)	NR	[70]
Gypenosides	100 or 300 mg/(kg · d)	C57BL/6J (male)	6	12	22–24	Y	40-50	NR	HFD	*n* = 8	BAT: *Ucp1, Cidea, Pgc1a* (high dose) (qRT-PCR); UCP1 (both doses), PGC1A, PRDM16 (high dose) (inmunoblot)	NR	[108]
Histidine	1, 2.5, and 5%	Wistar Rat (male)	6	1	23 ± 2	Y	NR	Y	CE-2, CLEA Japan	*n* = 6	BAT: UCP1 (5% dose, northenblot)	NR	[109]
Inorganic Nitrate	0.35, 0,7 and 1.4 mMol/(L · d)	Wistar Rats (male)	6	3	NR	Y	NR	Y	STD	*n* = 6	NR	iWAT:*Ucp1, Tbx1, Tmen26, Cidea* (qRT-PCR); UCP1, PGC1A and CPT1 (inmunoblot)	[110]

Jinlida	3.8 g/(kg · d)	C57BL/6J mice (male)	6-7	15	22	Y	NR	Y	HFD	*n* = 15	BAT: *Ucp1, Dio2, Prdm16, Evolv3* (qRT-PCR)	NR	[111]
Kaempferia parviflora extract	0.5 or 1% diet	C57BL/6J mice (male)	NR	7	NR	NR	NR	NR	HFD	*n* = 8	BAT: UCP1 (1% dose, inmunoblot)	NR	[112]
Leucine	1.5% wt/vol	C57BL/6J mice (male)	20	10	22	Y	NR	Y	HFD	*n* = 5	BAT: No effect on *Ucp1* (qRT-PCR)	NR	[50]
Leucine	1.5% wt/vol	C57BL/6J mice (male)	15	3	22	Y	NR	Y	STD	*n* = 5	BAT: No effect on *Ucp1* (qRT-PCR)	iWAT: *Ucp1* (qRT-PCR)	[51]
Leucine	1.5% or 3%	C57BL/6J mice (male)	8	24	22 ± 2	Y	60	Y	HFD	*n* = 14	BAT: UCP1, PGC1A (inmunoblot)	WAT: UCP1, PGC1A (inmunoblot)	[52]
Limonoid 7-Deacetoxy-7-oxogedunin (Carapa guianensis)	20 mg/(kg · d)	C57BL/6J mice (male)	6	7	24	Y	NR	Y	LFD and HFD	*n* = 10	BAT: *Ucp1, Ucp2, Ucp3, Prdm16, Cidea, Pgc1a* (HFD, qRT-PCR)	vWAT: *Ucp1* (qRT-PCR)	[113]
Luteolin	0.1%	C57BL/6 mice (male)	5	12	22 ± 2	Y	NR	Y	HFD	*n* = 12	BAT: *Ucp1, Pgc1a, Cidea*. No changes for *Prdm16, Elovl3* (qRT-PCR); UCP1	sWAT: *Ucp1, Tmem26, Cd137, Pgc1a, Cidea, Elovl3*(qRT-PCR), no effect	[71]

											(inmunoblot and immunostaining)	on *Prdm16* (qRT-PCR); UCP1 (inmunoblot and immunostaining)	
Luteolin	0.003% wt/wt	C57BL/6 mice (male)	5	16	24	Y	NR	NR	HFD	*n* = 10	BAT: UCP1 (immunostaining)	eWAT: no effect on *Ucp1, Cox8b* (only *Cidea*, qRT-PCR)	[72]
Lyophilized Maqui (Aristotelia chilensis) Berry	20 mg/mL	C57BL/6J mice (male)	4	16	22 ± 1	Y	NR	Y	HFD	*n* = 14	NR	sWAT: *Ucp1, Pgc1a, Prdm16, Dio2* (qRT-PCR)	[114]
Matured hop bittering components	0,0025% to 1% (week 1) until 0,2% (week 2 to 9)	C57BL/6J mice (male)	6	9	NR	Y	NR	Y	HFD	*n* = 12	BAT: *Ucp1, Pgc1a,Prdm16* (qRT-PCR)	NR	[115]
Melinjo (Gnetum gnemon L.) seed extract	1% wt/wt	C57BL/6J mice (male)	3	17	22	Y	NR	NR	HFD	*n* = 6	BAT: UCP1 (inmunoblot)	iWAT and gWAT: no effect on UCP1 (inmunoblot)	[116]
Menthol	0.5%	C57BL/6J mice (male)	6-8	28	21-23	Y	NR	Y	HFD	*n* = 4	BAT: UCP1 (inmunoblot)	NR	[53]

Menthol	1%	C57Bl/6 mice (male)	6	12	22-24	Y	50-60	Y	HFD	*n* = 8	NR	sWAT: *Ucp1, Pgc1a, Prdm16* (qRT-PCR); eWAT: *Ucp1, Pgc1a, Prdm16* (qRT-PCR)	[54]
Menthol	50 or 100 mg/(kg · d)	Swiss Albino mice (male)	7-8	12	22 ± 2	Y	55 ± 5	Y	HFD	*n* = 6-8	BAT: *Ucp1, Pgc1a, Prdm16, Cidea, Tbx1, Bmp4* (qRT-PCR)	vWAT: *Ucp1, Pgc1a, Prdm16, Cidea, Tbx1* (qRT-PCR). No effect on *Tmem* or *Cd137* (qRT-PCR)	[55]
Momordica charantia	1 and 1,25%	Sprague–Dawley rats (male)	9	6	22	Y	NR	Y	HFD	*n* = 8	BAT: *Ucp1* and *Pgc1a* (RT-PCR); UCP1 (inmunoblot)	NR	[117]
Monosaccahrides (D-dextrose, D-fructose and D-galactose)	diets received an amount of monosaccharide equal in energy to the food ration twice the dietary energy of animals fed food alone	Sprague–Dawley rats (male)	8	1	21 ± 2	14:10 light;dark cycle	NR	NR	STD	*n* = 8	BAT: *Ucp1* (qRT-PCR)	NR	[38]
Mulberry leaves	20% mulberry leave powder wt/wt	C57BL/6J (male)	4	13	22 ± 2	Y	55% ± 10	NR	HFD	*n* = 6	BAT: UCP1, PGC1A, PGC-1B,PPARg (inmunoblot)	NR	[118]

Myrciaria dubia	200 mg/(kg ·d)	C57BL/6J mice (male)	10	8	NR	Y	NR	Y	HFHSD	*n* = 12	BAT: *Ucp1, Pparg2, Dio2* (qRT-PCR)	iWAT: *Ucp1, Pgc1a* (qRT-PCR), no changes nor Dio2) (qRT-PCR); eWAT: No changes on *Ucp1, Pparg2* (qRT-PCR)	[119]
Nigella sativa extract	200 mg/(kg ·d) (NS 200); 300 mg/kg ·d (NS 300) 100 mg/(kg ·d)	NR	6	4	22 ± 2	Y	NR	Y	HFD	*n* = 10	BAT: UCP1 (NS 200 and 300; inmunoblot)	NR	[120]
Nitzschia laevis extract	10 or 50 mg/(kg ·d)	C57BL/6J mice (male)	11	8	25 ± 2	Y	NR	Y	HFD	*n* = 12	BAT: *Ucp1* (both), *Pgc1a* (high dose group) (qRT-PCR)	NR	[121]
Non-nutritive sweetener (NNS) and sucrose	4% drinking water NNS (99% eritriol, 1% aspartame), 33% sucrose drinking water	C57BL/6J mice (male)	8	4	21 ± 1	Y	55 ± 5	Y	HFD	*n* = 5	BAT: Downregulation of *Ucp1* (both), but no effect on Pgc1α (qRT-PCR)	NR	[40]
Octacosanol and policosanol	60 mg octosanol/(kg ·d) + 60 mg policosanol/ (kg ·d)	C57BL/6 mice (male)	10	4	NR	Y	NR	Y	HFD	*n* = 5	BAT: *Ucp1, Ppargc1a, Elovl3* (qRT-PCR); UCP1 (inmunoblot)	iWAT: no effect for Ucp1 (qRT-PCR). *Ppargc1a, Cidea, Prdm16* (qRT-PCR)	[122]

Oleuropein aglycone	1. 2 or 4 mg/(kg · d)	Sprague–Dawley rats (male)	7	4	22–24	Y	50	Y	HFD	*n* = 6-7	BAT: UCP1 (0,1 and 0,2%, inmunoblot)	NR	[73]
Oleuropein aglycone	1 g/(kg diet · d)	Sprague–Dawley rats (male)	4	4	22–24	Y	50	Y (tap water) 11:00 to12:00 h, 6 times/ wk food	HFD	*n* = 7	BAT: UCP1 (inmunoblot)	NR	[74]
Olive oil	40% of fat diet by olive oil	Wistar rats (male)	NR	4	22 ± 2	Y	50%	Y	HFD	*n* = 10	BAT: *Ucp1* (qRT-PCR); UCP1 (inmunoblot)	NR	[76]
Olive oil (regular, R; extra virgin, EV)	300 g/kg diet (R and EV)	Sprague–Dawley rats (male)	NR	4	22 - 24	Y	50%	Y	HFD	*n* = 6-7	BAT:UCP1 (EV, inmunoblot)	NR	[75]
Omija fruit extract	500 mg/(kg · d)	C57BL/6J mice (male)	5	16	22 ± 2	Y	40 ± 1	Y	HFD	*n* = 10	NR	iWAT: no effect on *Ucp1*, *Pgc1a* (qRT-PCR), upregulation of *Cidea, Cox8b* (qRT-PCR)	[123]
Oolang (O), black (B) and pu-erh (P) tea extract	2 g/100 mL	IRC mice (male)	5 and 7	1	23 ± 2	Y	NR	Y	STD	*n* = 4-5	NR	mWAT: *Ucp1* (B, O, qRT-PCR); UCP1 (B, O, inmunoblot)	[124]

Out of season orange	100 mg/(kg · d) from the northern hemisphere (ON) or from the southern hemisphere (OS); ONSD (Orange North Hemisphere Short Day), OSSD (Orange South Hemisphere Short Day), VHSD (Vehicle Short Day), ONLD (Orange North Hemi-sphere Long Day), OSLD (Orange South Hemisphere Long Day), and VHLD (Vehicle Long Day)	Fischer 344/IcoCrl rats (male)	8.5	10	22	Long day (LD): 18 h light: 6 h dark; short day (SD):6 h light: 18 h dark	55	Y	STD	*n* = 6	BAT: *Ucp1* (OSSD, qRT-PCR), no effect on UCP1 (inmunoblot)	iWAT: no effect on *Ucp1, Prdm16* between OSSD and VHSD (qRT-PCR)	[125]

Panax ginseng	200 mg/(kg · d) of White ginseng (WG), 3 h-steamed ginseng (SG-3), 6 h-steamed ginseng (SG-6), and 9 h-steamed ginseng (SG-9).	Sprague–Dawley rats (male)	7	5 days (shot term) or 4 weeks (long term)	24 ± 2	Y	60 ± 10	Y	?	*n* = 8	BAT: no effect on UCP1 nor Pgc1α (both terms, all groups, qRT-PCR)	NR	[126]
Pentamethylquercetin (PMQ)	0.4% g/g	C57BL/6 mice (male)	4-5	17	22 ± 2	Y	55 ± 5	NR	HFD	*n* = 10	NR	eWAT:*Ucp1, Cidea, Pgc1a* (qRT-PCR)	[36]
Phaeodactylum tricornutum extract	100 or 300 mg/(kg · d)	C57BL/6J mice (male)	6-8	4	22º	Y	NR	Y	HFD	*n* = 6	BAT: UCP1 (both doses, immunostaining and inmunoblot)	iWAT:*Ucp1* and *Cpt1a*; eWAT: no effect in *Ucp1* and *Cpt1a*	[127]
Phosphate	1.2%	Sprague-Dawley (male)	11	10	22 ± 2	Y	NR	Y	AIN-93G modified diet	*n* = 6	BAT:Ucp1 (qRT-PCR)	NR	[128]
Phyllodulcin or Stevioside (sweeteners)	Stevioside at 40 mg/(kg · d) (SVS), phyllodulcin at 20 mg/(kg · d) (P 20), or	C57BL/6J mice (male)	11	7	22 ± 2	Y	50% ± 5	Y	HFD	*n* = 12	NR	sWAT: *Ucp1, Prdm16, Pgc1a* (P40, qRT-PCR)	[42]

	phyllodulcin at 40 mg/(kg · d) (P 40)												
Phytol	500 mg/(kg · d)	C57BL/6J mice (male)	5	7	NR	Y	NR	Y	HFD	*n* = 12	NR	iWAT: *Ucp1, Prdm16, Pgc1a* (qRT-PCR)	[129]
Plantago asiatica extract	1.44 g/(kg · d)	C57BL/6 mice (male)	16	2	22 ± 2	Y	60-65	Y	HFD	*n* = 8	BAT: *Ucp1* (qRT-PCR)	NR	[130]
Platycodon grandiflorus Root Ethanol Extract	5% wt/wt	C57BL/6J mice (male)	5	12	NR	NR	NR	NR	HFD	*n* = 10	BAT: *Ucp1, Prdm16, Cidea, Cd137* (qRT-PCR); UCP1 (immunostaining)	iWAT: *Ucp1, Prdm16, Cidea*, Cd137 (qRT-PCR); UCP1 (immunostaining)	[77]
Platycodon grandiflorus Root Extract	5% wt/wt	C57BL/6J mice (male)	5	12	24	Y	NR	NR	HFD	*n* = 10	NR	eWAT: *Ucp1, Pgc1a* (qRT-PCR)	[78]
Psoralea corylifolia L. (Prenylated flavonoid-standardized extract)	0,2 and 0.5% wt/wt	C57BL/6J mice (male)	7	14	23 °C ± 1	Y	50	Y	HFD	*n* = 10	BAT: *Ucp1,Pparg, Pgc1a, Cidea, Prdm16, Dio2, Cox8b* (qRT-PCR)	sWAT:*Ucp1, Pparg Pgc1a, Cidea, Prdm16, Pgc1b, Cox8b* (qRT-PCR)	[131]

Puerariae flower extract (and isoflavone-rich extract)	5% diet (whole extract) and 1,355% from yield extraction (isoflavone-rich extract)	C57BL/6J mice (male)	7	2	NR	Y	NR	Y	HFD	*n* = 8	BAT: *Ucp1* (qRT-PCR)	NR	[56]
Puerariae flower extract (and isoflavone-rich extract)	5% diet (whole extract) and 1.355% from yield extraction (isoflavone-rich extract)	C57BL/6J mice (male)	7	6	NR	Y	NR	Y	HFD	*n* = 7-8	BAT: UCP1 (immunostaining)	NR	[57]
Puerariae lobatae root extract	0.4 and 0.8 g/(kg · d)	NR	16	2	NR	NR	NR	NR	HFD	*n* = 5	BAT:*Ucp1* (qRT-PCR)	iWAT: *Ucp1* (0,4 dose, qRT-PCR)	[58]
Quercetin	0.01% wt/wt	C57BL/6J mice (male)	9	12	NR	NR	NR	Y	HFD	*n* = 8-10	BAT: No effect on *Ucp1, Pgc1a, Elolvl3* (qRT-PCR)	sWAT:*Ucp1* and *Elolv3* (no changes for *Prdm16, Cidea* nor *Pgc1a*) (qRT-PCR)	[34]
Quercetin	0.05% wt/wt	C57BL/6J mice (male)	9	9	22 ± 2	Y	55-60	Y	HFD	*n* = 6	BAT: UCP1 (inmunoblot)	sWAT: *Ucp1, Tmem26* (qRT-PCR)	[33]

Quercetin	0.36% and 0.72%	Sprague–Dawley rats (male)	6	8	18–24	Y	50–60	Y	HFD	NR	NR	sWAT: *Ucp1* (0.36% dose, qRT-PCR)	[37]
Quercetin (onion peel extract)	0.5%	C57BL/6J mice (male)	4	8	22	Y	NR	Y	HFD	*n* = 6	NR	sWAT: *Cidea* (no changes for *Ucp1, Pgc1a, Prdm16,* Tbx1) (qRT-PCR); rWAT: *Ucp1, Pgc1a, Prdm16, Cidea* (no changes for Tbx1)(qRT-PCR)	[35]
Quercetin (Q) + Resveratrol (RSV)	15 mg/(kg · d) (RSV), 30 mg/(kg · d) (Q), or 15 mg/(kg · d) (RSV) + 30 mg/(kg · d) (Q) (R+Q)	Wistar Rat (male)	5	6	22 ± 2	13 h light:dark cycle	NR	Y	HFHSD	*n* = 9	BAT: UCP1 (RSV, R+Q, inmunoblot); *Cidea* (RSV, qRT-PCR)	pWAT: no changes for *Ucp1, Prdm16, Cd137, Tmem26* (changes for *Cidea* in R+Q, qRT-PCR)	[132]
Quercetin and Isoquercitrin (EMIQ)	quercetin or EMIQ at 0.02%, 0.1% and 0.5% diet	C57BL/6 mice (male)	5	2	23 ± 2	Y	NR	Y	STD	*n* = 4	NR	sWAT: UCP1, PRDM16, PGC1A (0,1% EMIQ, inmunoblot)	[32]
Raspberry	5%	C57BL/6J mice (male)	6	12	NR	NR	NR	NR	HFD	*n* = 8	NR	iWAT: *Ucp1, Cidea, Elvol3* (qRT-PCR); UCP1 (inmunoblot)	[80]

Raspberry	5%	C57BL/6J mice (male)	9	10	23 ± 2	Y	NR	Y	HFD	*n* = 6	BAT: *Ucp1, Prdm16, Pgc1a, Cidea* (qRT-PCR); UCP1, PRDM16 (inmunoblot); UCP1 (immunostaining)	iWAT: *Ucp1, Prdm16, Pgc1a, Tbx1, Tmem26, Cd137* (no *Cidea*, qRT-PCR); UCP1, PRDM16 (inmunoblot); UCP1 (immunostaining)	[79]
Resveratrol	25 mg/d	NR (male mice)	11	6	NR	Y	NR	Y	STD	*n* = 4-5	BAT: *Ucp1, Pgc1a* (qRT-PCR)	NR	[17]
Resveratrol	30 mg/(kg · d)	Wistar Rat (male)	6	6	22 ± 2	Y	NR	Y	HFD (high sucrose)	*n* = 8	BAT: UCP1 (inmunoblot); *Sirt1, Pgc1a*, (qRT-PCR)	NR	[18]
Resveratrol	4 kg/(kg · d)	NR (male mice)	NR	8	NR	NR	NR	Y	STD	*n* = 8	BAT: *Ucp1, Bmp7* (no changes for *Cidea, Prdm16*; qRT-PCR)	NR	[19]
Resveratrol	0.1% wt/wt	cd1 mice (female)	22	4	NR	Y	NR	NR	HFD	*n* = 6	NR	iWAT: *Ucp1, Tme26, Cd137, Tbx1, Cidea, Prdm16* (qRT-PCR); UCP1, PRDM16 (inmunoblot); UCP1 (immunostaining)	[20]

Resveratrol	10 mg/(kg · d)	OLETF rats (male)	7	27	23°C ± 2	Y	55% ± 5	Y	STD and HFD	*n* = 7-8	BAT: UCP1 (HFD, inmunoblot)	NR	[22]
Resveratrol	0.1% wt/wt	cd1 mice (female)	22	4	NR	Y	NR	Y	HFD	NR	BAT: UCP1, PRDM16 (inmunoblot); UCP1 (immunostaining)	NR	[23]
Resveratrol	0.5% resveratrol (RES) or 0.1% or 0.5% oxyresveratrol (LOXY, HOXY)	C57BL/6 mice (male)	6	10	25°C ± 1	Y	50	Y	HFD	*n* = 10	NR	sWAT: UCP1, PRDM16, PGC1A (inmunoblot)	[24]
Resveratrol	30 mg/(kg · d)	Wistar Rat (male)	7	6	22 ± 2	Y	NR	Y	HFD (high sucrose)	*n* = 9	BAT: *Ucp1, Pgc1a* (qRT-PCR);	NR	[21]
Rojal Jelly (RJ) or Bee Larva Powder (BL)	5% diet RJ or BL	C57BL/6J mice (male)	4	17	22 ± 2	Y	NR	Y	HFD	*n* = 11	BAT: no differences for *Ucp1* nor *CoxIV* (qRT-PCR); UCP1 and COXIV (only RJ, inmunoblot)	sWAT and iWAT: no effect on *Ucp1* or *CoxIV* (qRT-PC, inmunoblot)	[133]
Rose hip	295 g/diet	C57BL/6J mice (male)	9	13	22	Y	NR	Y	HFD	*n* = 12	BAT: no effect on *Ucp1, Pgc1a ,Cidea, Prdm16, Elovl3* (qRT-PCR)	sWAT: *Ucp1, Bmp7, Pgc1a, Cidea* (qRT-PCR)	[134]

Rubi Fructus *(Rubus coreanus)*	100 mg/d	C57BL/6J mice (male)	9	10	NR	Y	NR	NR	HFD	*n* = 5	BAT: *Ucp1, Cidea, Pgc1a* (qRT-PCR)	NR	[135]
Rutin	1 mg/mL	C57BL/6J mice (male)	3	10	NR	NR	NR	Y	HFD	*n* = 10	BAT: *Ucp1* (qRT-PCR); UCP1 (inmunoblot)	NR	[136]
Sesaminol diglucoside	20 mg/(kg · d)	C57BL/6J mice (male)	8	2	22 ± 2	Y	NR	NR	HFD	*n* = 6	BAT: *Ucp1* (qRT-PCR); UCP1 (inmunoblot)	NR	[137]
Soy Isoflavones	600 µg isoflavones/ g · diet	Long Evans rats (male)	7	4	NR	11-hour dark 13-hour light	NR	NR	STD	*n* = 8	BAT: *Ucp1* (qRT-PCR)	NR	[138]
Sucrose and high starch	35% sucrose (58,2 % CHO) or high starch (74,3% CHO)	C57BL/6J mice (male)	8	15	22-24	Y	NR	NR	STD	*n* = 5	BAT: *Ucp1* (no effect for *Dio2*, qRT-PCR)	WAT: *Ucp1* (qRT-PCR), no differences for *Cd137, Tbx1, Cidea* (qRT-PCR)	(58)
Sweeteners (sucrose, fructose, glucose, brown sugar, steviol glycosides, honey)	Sucrose (S), fructose (F), glucose (G), brown sugar (BS), honey (H), steviol glycosides, plus sucrose (SV) at 10%; steviol	Wistar rats (male)	5	17	NR	Y	NR	Y	HFD	*n* = 6	BAT: *Ucp1* (S, BS, and H, qRT-PCR)	NR	[39]

	glycosides (SG) at 2.5% and sucralose (SU) at 1.5% diet											
Tea Catechins	100 mg/(kg · d)	Sprague Dawley rats (male)	8	30 or 45 days	22	Y	NR	NR	HFD	*n* = 10	BAT: *Ucp1* (qRT-PCR)	vWAT and sWAT:*Ucp1* (RT-PCR)	1
Tea catechins	0.5 g/(100 g · diet)	Sprague Dawley rats (male)	18	8	23–25	Y	NR	Y	STD and HFD	*n* = 8	BAT: *Ucp1* (STD, qRT-PCR)	NR	2
Tea catechins	7.7 g/(kg · d)	C57BL/6J mice (male)	4	16	NR	NR	NR	NR	HFD	*n* = 22	NR	eWAT: *Ucp1* (qRT-PCR)	3
Tea catechins	77.5 mg/kg · d or 155 mg/(kg · d)	Sprague Dawley rats (male)	6	8	22-24	Y	40-60	NR	HFD	*n* = 10	NR	eWAT: *Ucp1, Cidea, Cpt1a, Pgc1a, Pparg, Prdm16, Bmp7* (qRT-PCR); UCP1 (inmunoblot)	4
Tea catechins	0.5%	C57BL/6J mice (male)	9	8	NR	Y	NR	Y	HFD	NR	NR	sWAT: UCP1 (inmustaining). *Pgc1a, Prdm16, Cited1* (qRT-PCR)	5

Thea flavins	10 mg/kg	IRC mice (male)	8-11	Acute study	23–25	Y	NR	Y	STD	*n* = 8	BAT: *Ucp1, Pgc1a* (after 2 and 5 h ingestion; qRT-PCR)	NR	[6]
Thymoquinone	Thymoquinone 100 mg/kg	C57 mice (male)	6	4	22 ± 2	Y	NR	Y	HFD	*n* = 10	BAT: UCP1 (inmunoblot)	NR	[120]
Undaria pinnatifida	400 mg/kg	Wistar rats (male)	7	8	24 ± 2	Y	40-60	Y	HFD	*n* = 6	NR	rWAT: *Ucp1* (qRT-PCR)	[68]
Vanillic Acid	10, 100, 1000 mg/(kg · d)	C57BL/6J mice (male)	9	6	NR	NR	NR	NR	HFD	*n* = 5	BAT: UCP1 and PGC1A (inmunoblot)	NR	[139]
Vitamin A	129 mg retinol/kg · diet	F-344 x BN rats (male)	22	8	26	Y	NR	Y	IAMS Company	*n* = 6-7	BAT: *Ucp1* (qRT-PCR)	NR	[81]
Vitamin A	320 mg retinyl palmitate · kg diet	C57BL/6J mice (male)	4	18	22	Y	NR	Y	HFD	*n* = 4	BAT: UCP1 downregulation (inmunoblot)	NR	[82]
Wheat gluten	4.5%	C57BL/6 mice (male)	8	8	26 ± 2	Y	NR	Y	HFD	*n* = 4	BAT: *Ucp1, Pgc1a* downregulation, no effect on *Prdm16, Bmp7* (qRT-PCR)	sWAT: *Ucp1, Prdm16* downregulation; no changes for *Pgc1a,*	[140]

												Bmp7 (qRT-PCR)	
α/γ-Tocopherol	0.8 g/kg diet	SD-IGS rats (male)	5	8	23 ± 2	Y	55 ± 5	NR	HFD	*n* = 5-6	NR	WAT: *Ucp1*, *ta* (α-tocopherol, qRT-PCR)	[141]
α-Monoglucosyl Hesperidin (αMH) and Hesperidin (H)	5 or 10 g/(kg diet · d) (α-Monoglucosyl Hesperidin) 8,1 g/kg/diet/d hesperidin	C57BL/6J (male)	5	4	23 ± 2	Y	NR	Y	AIN-93G	*n* = 8	BAT: no changes for UCP1 (inmunoblot)	iWAT: UCP1 (High αMH, inmunoblot and immunostaining); eWAT: no effect on UCP1 (immunostaining)	[142]

Values are mean ± SD, otherwise stated. Adbr3, beta-3 adrenergic receptor; Ampk, AMP-activated protein kinase; BAT: brown adipose tissue; Bmp4, bone morphogenetic protein 4; Bmp8b, bone morphogenetic protein 8b; Cd137, cluster of differentiation 137; Cidea: cell death activator A; Cited1, Cbp/p300-interacting transactivator 1; CoxIV, cytochrome c oxidase subunit 4; Cox7a, cytochrome C oxidase subunit 7a; Cox8b, cytochrome C oxidase subunit 8b; Dio2, iodothyronine deiodinase 2; Elovl3, fatty acid elongase 3; eWAT, epididymal WAT; gWAT, gonadal WAT; HFD, high fat diet; HFSD, high fat and high sugar diet; iWAT, inguinal WAT; LFD, low fat diet; Mapk, mitogen-activated protein kinase; mWAT, mesenteric WAT; Pgc1a, peroxisome proliferator-activated receptor-gamma coactivator 1 alpha; Ppara, peroxisome proliferator-activated receptor alpha; Ppargc1b, peroxisome proliferator-activated receptor gamma coactivator 1-beta; Prdm16, PR domain containing 16; pWAT: perirenal WAT; rWAT: retroperitoneal WAT; Sirt1, sirtuin 1; STD: standard diet; sWAT: subcutaneous WAT; Tbx1, T-Box transcription factor 1; Tmem26, transmembrane protein 26; UCP1, uncoupling protein 1; vWAT, visceral WAT; WAT, white adipose tissue; WD, western diet.

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RESULTS AND DISCUSSION

SECTION II

STUDY V: Dihydrocapsiate does not increase energy expenditure nor fat oxidation during aerobic exercise in men with overweight/obesity: a randomized, triple-blinded, placebo-controlled, crossover trial

(Under review – Clinical Nutrition)

ABSTRACT

Background: Prior evidence suggests that capsinoids ingestion may increase resting energy expenditure (EE) and fat oxidation (FATox) in humans, yet whether they can modulate those parameters during exercise conditions remains poorly understood.

Aim: To determine the effects of dihydrocapsiate (DHC) ingestion on EE and FATox during an acute bout of aerobic exercise at FATmax intensity (the intensity that elicits maximal fat oxidation during exercise [MFO]) in men with overweight/obesity.

Methods: A total of 24 sedentary men (age = 40.2 ± 9.2 years-old; body mass index = 31.6 ± 4.5 kg/m2) participated in this randomized, triple-blinded, placebo-controlled, crossover trial (registered under ClinicalTrials.gov Identifier no. NCT05156697). On the first day, participants underwent a submaximal exercise test in a cycloergometer to determine their MFO and FATmax intensity during exercise. After 72 hours had elapsed, the participants returned in 2 further days (≥ 72 hours apart) and performed a 60 min steady-state exercise bout (i.e., cycling at their FATmax, constant intensity) after ingesting either 12 mg of DHC or placebo; these conditions were randomized. Respiratory gas exchange was monitored by indirect calorimetry. Serum markers concentrations (i.e., glucose, triglycerides, and non-esterified fatty acids (NEFAs), and skin temperature, thermal perception, heart rate and perceived fatigue were assessed.

Results: There were no significant differences (P>0.05) between DHC and placebo conditions in the EE and FATox during exercise. Similarly, no significant changes were observed in glucose, triglycerides or NEFAs serum levels, neither in the skin temperature or thermal perception across conditions. Heart rate and perceived fatigue did not differ between conditions.

Conclusion: Our findings do not support the use of DHC as a tool to further increase EE and FATox during exercise at FATmax in men with overweight/obesity.

BACKGROUND

Poor nutritional habits, sedentarism and physical inactivity are among the foremost modifiable risk factors related to cardiovascular disease (CVD) and all-cause mortality [1]. Optimizing any of these components is key to improve cardiometabolic health and weight loss success in the long term [2]. In this regard, the use of nutraceuticals and natural food ingredients aiming to increase energy expenditure (EE) and fat oxidation (FATox) has attracted a great deal of attention over the past decade, especially among overweight/obese individuals [3].

The ingestion of capsaicin has been shown to elicit significant increases in EE and FATox in humans [4]. Nonetheless, tolerance to capsaicin varies between individuals, and its ingestion in high doses could lead to pain, swelling, and gastrointestinal problems [5]. Capsinoids, which include capsiate, dihydrocapsiate (DHC), and nordihydrocapsiate, are significantly less pungent chemical analogs of capsaicin [6]. The ingestion of capsaicin and capsinoids activates the transient receptor potential vanilloid subfamily member 1 (TRPV1) in the gastrointestinal tract [7]. In fact, ingestion of CH-19 sweet pepper, a capsinoid-rich variety of pepper, increases core body temperature [8,9] and oxygen consumption [8], which is likely to be explained by increased sympathetic nervous activity and catecholamines levels [9,10]. The use of purified capsinoids has been suggested to yield similar effects, although this needs to be further examined [11–13].

Given that endurance training programs effectively help in weight loss and maintenance, and cardiometabolic risk management in adults with overweight/obesity [14], the use of capsinoids as potential coadjutants to increase EE or FATox during exercise is of particular interest. Thus far, only one study has investigated the effect of capsinoids ingestion on EE and FATox during aerobic exercise in healthy active young men, showing a lack of effect [15]. Importantly, this study was conducted in normal-weight adults, yet it appears that capsinoids intake could enhance EE and FATox particularly in individuals with overweight/obesity [16]. Therefore, we hypothesized that DHC ingestion could further increase the EE and FATox during an exercise bout designed to elicit maximal fat oxidation (MFO) in sedentary men with overweight/obesity.

The aim of the present study was to investigate the acute effects of the ingestion of 12 mg of DHC (the highest dose approved by the European Food Safety Authority, EFSA), on EE and FATox during a bout of 60 min of aerobic exercise at FATmax intensity in men with overweight/obesity. As a secondary aim, we investigated the effects of DHC on other physiological parameters, such as blood markers, skin temperature, thermal perception, heart rate and perceived fatigue.

METHODS

Study subjects and experimental design

The current work was conducted within the framework of the ACTIFOX (ACTIvating Fat OXidation through capsinoids) study, a randomized, triple-blinded, placebo-controlled, crossover trial designed to determine the effect of DHC on EE and FATox during aerobic exercise (ClinicalTrials.gov ID: NCT05156697). A total of 24 men with overweight or obesity (40.2 ± 9.2 years-old, body mass index (BMI) >25 kg/m^2) participated in the study. The caption flow of the participants to the ACTIFOX study is depicted in **Fig. 1**

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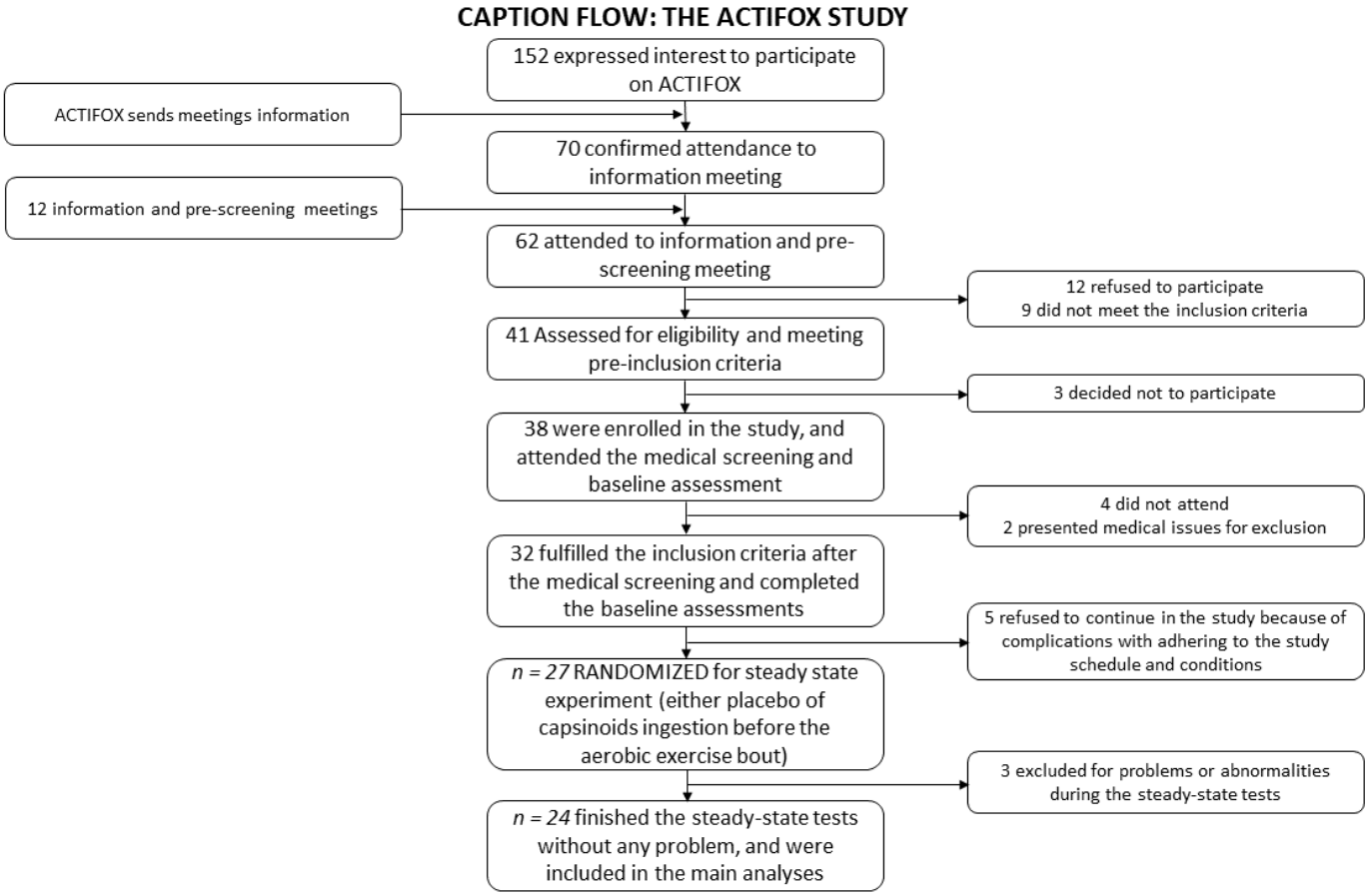

Figure 1. Caption flow of the ACTIFOX study.

Inclusion criteria were to be male and 18-55 years-old, to be sedentary (subjects reported <20 min moderate to vigorous physical activity on <3 days/week), to be non-smoker, not being under medication that could affect energy metabolism, and to have a stable body weight over the preceding 3 months (<3 kg change). Exclusion criteria were: having been diagnosed with diabetes, hypertension or any medical or cardiometabolic condition(s) that could interfere with or be aggravated by exercise, presenting a family history of CVD, to have an abnormal

electrocardiogram, regular and high consumption of spicy foods, and being frequently exposed to cold temperatures (*e.g.*, indoors/outdoors workspace with low-temperatures, such as cold-storage works, ski/snow monitors, etc.). All participants gave their written informed consent. The study protocol and design were approved by the Human Research Ethics Committee of the University of Granada (n°839/CEIH/2019) and the Servicio Andaluz de Salud and adhered to the tenets of the Declaration of Helsinki as revised in 2013. The study was carried out in Granada (Spain), from October 2019 to March 2020.

Procedures

Overall procedures

Fig. 2 shows the overall-design of the study. Data for each participant were collected over 4 visits at the research centre within 3 weeks.

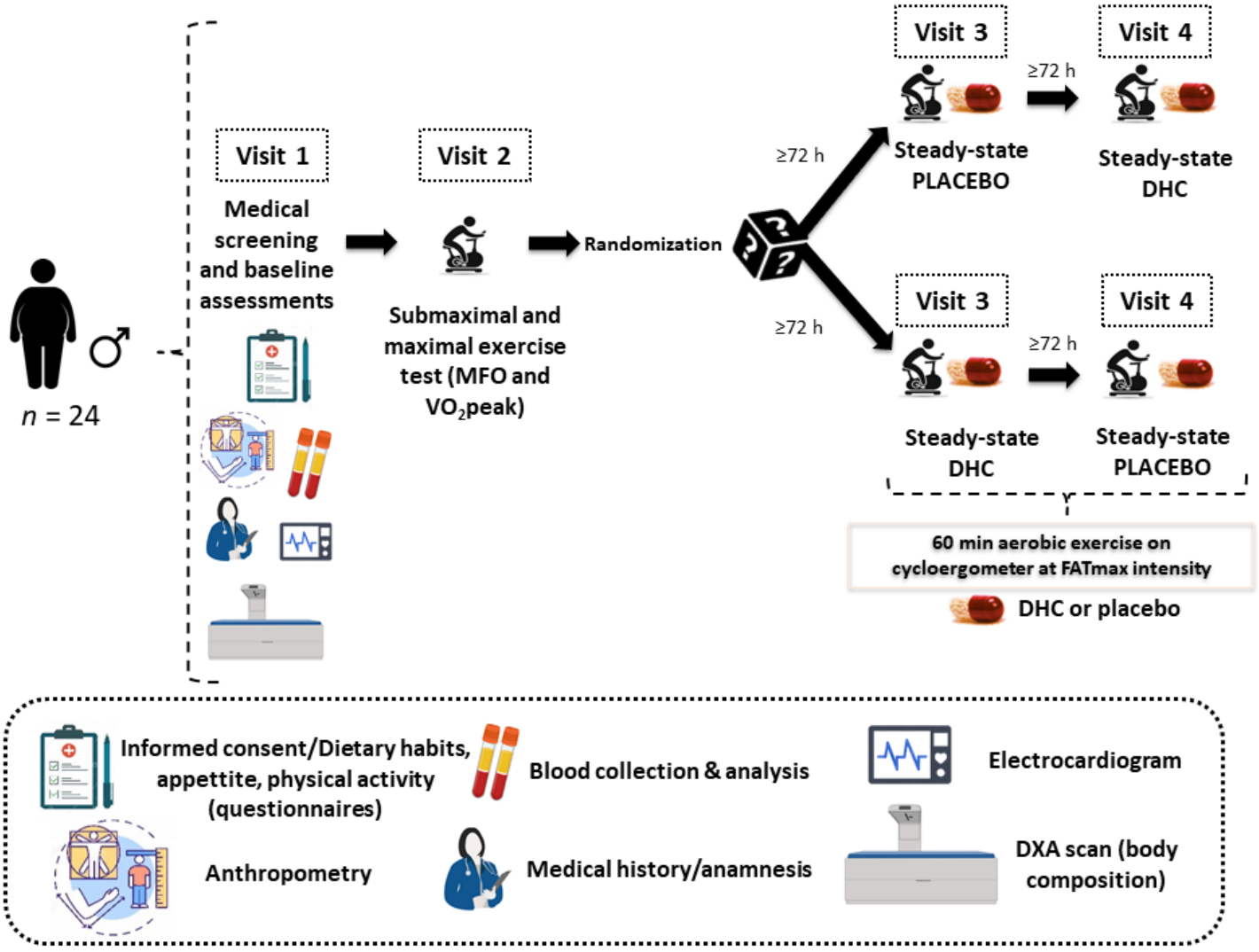

Figure 2. Overview of the ACTIFOX study procedures. Abbreviations: DHC: dihydrocapsiate, DXA: dual energy X-ray absorptiometry test, MFO: maximal fat oxidation, VO_2peak: peak oxygen consumption.

Participants were asked to confirm having commuted to the research center by car, bus, tram, or motorcycle, having slept as usual, having refrained from stimulant beverages within 24 h, and having avoided any moderate or vigorous physical activity within 24 h and 48 h (respectively). Other specific pre-experimental conditions were established for each visit are detailed in the following sections.

Briefly, in the first visit, sociodemographic and lifestyle data were registered, a medical screening was performed, blood samples were collected, and anthropometry and body composition measures were taken. On the second visit, the MFO during exercise and cardiorespiratory fitness (VO_2 peak) were respectively assessed through a submaximal exercise test coupled to a maximal effort test. On visits third and fourth, participants performed a 60 min steady-state exercise bout on a cycloergometer at FATmax intensity (i.e., at the intensity at which MFO is elicited) after having ingested either 12 mg of DHC or placebo. The conditions (DHC or placebo) on visits 3 and 4 were randomized. The washout period between visits 3 and 4 was ≥72 h. Of note, all exercise tests took place at a strictly controlled temperature of 22-23 ^{o}C, given that environmental temperature largely influences EE and FATox [17].

Medical screening, sociodemographic data collection, and anthropometry and body composition assessments

On the first visit, participants arrived at the research center at 08:00 h, in fasting conditions (8 h). They were informed about the study protocols and gave their oral and written informed consent to participate in the study. Sociodemographic data and details related to their dietary habits (including pungent consumption), appetite, physical activity levels, sleep, and other lifestyle habits were recorded by questionnaires. Dietary intake was assessed using a previously validated food frequency questionnaire (FFQ) and three 24 h-recalls undertaken on three non-consecutive days, as previously described [18]. Afterwards, a medical doctor conducted an anamnesis to ensure that each participant was in suitable physical conditions to participate in the study, and to perform exercise. Subsequently, participants underwent an electrocardiogram in resting conditions conducted by an expert medical doctor. Systolic and diastolic blood pressure were also measured with an automatic sphygmomanometer (Omrom M2; Omron Healthcare, Kyoto, Japan). Measurements were repeated in 3 consecutives occasions and the average systolic and diastolic blood pressure calculated. Only participants presenting a non-risk medical history and normal electrocardiogram were allowed to participate in the study.

Blood samples were obtained from the antecubital vein in the morning (8.00–9.00 am), with subjects sat and in resting conditions. Blood samples were

collected in serum Vacutainer Tubes® (Vacutainer® SST™ II Advance tubes) and centrifuged following the manufacturer instructions. Afterwards, serum samples were sent to the hospital lab for the analysis of the analytes of interest.

Anthropometry and body composition assessments also took place on the first visit. Body weight and height were measured (no shoes, light clothing) using a model 799 Seca scale and stadiometer (Seca, Hamburg, Germany). Waist circumference was assessed twice at the minimum perimeter area with a measuring tape (mm precision), and the mean value calculated. For those participants with abdominal obesity, waist circumference was measured just above the umbilicus (horizontal plane). Body fat mass and percentage, lean body mass and visceral adipose tissue (VAT) mass were then measured by whole-body dual-energy X-ray absorptiometry (HOLOGIC, Discovery Wi, Marlborough, MA). Body mass, lean mass, and fat mass indexes were calculated as kg/m^2.

Exercise tests

On the second visit, individuals arrived at the research center, between 15:30 and 19:00. Participants confirmed having met the above stated pre-experimental conditions, as well as to arrive in fasting conditions (5-6 h) and having carried out a standardized diet that they were instructed to follow during the previous day. Then, they emptied their bladders, dressed standardized t-shirts and shorts, and entered in a quiet, warm (22-23 °C) room. A submaximal-graded exercise test (to determine the MFO) plus a maximum effort test (used to reach the VO_2peak) were performed employing an Ergoselect 200 cycle ergometer (Ergoline GmbH, Lindenstrasse, Germany). The exercise protocol, coupled to indirect calorimetry, started with a 3 min stage at 20 watts (W) as a warm-up, followed by increments of 20 W every 3 min, until respiratory exchange ratio (RER) was ≥1 at least for 30 s (as determined by indirect calorimetry) [19,20]. At this point, the maximal exercise protocol started (with no interruptions), implementing further increments of 20 W every 1 min until (i) volitional exhaustion was reached, or (ii) participants had to stop because of peripherical fatigue. Of note, the cycling power values (W) at which MFO happened for each individual were used as the target exercise intensity (i.e., FATmax) for the subsequent steady-state tests. Through the exercise test, participants' perceived fatigue was assessed using rating of the reported perceived exertion (RPE) scale, and heart rate was measured using a Polar RS800 heart-rate monitor (Polar Electro Inc., Woodbury, NY, USA). Respiratory gas exchange was monitored with a CPX Ultima CardioO2 system (Medical Graphics Corp., St Paul, MN) with a facemask model 7400 (Hans Rudolph Inc., Kansas City, MO), and a preVent™ metabolic flow sensor (Medical Graphics Corp.) [21]. Oxygen consumption (VO_2) was measured using a galvanic fuel cell and carbon dioxide production (VCO_2) was assessed using a non-

dispersive infra-red sensor [21]. According to the manufacturer's recommendations, the gas analyzer was calibrated using standard gas concentrations immediately before each test. Of note, an experienced medical doctor continuously monitored the hearth rhythm and electrical activity through the whole exercise test by means of an electrocardiogram, being the test stop if required by the doctor based on medical criteria.

Steady-state exercise bout

On the third visit (≥72 h after the second day), and on the fourth visit (≥72 h later after the third visit to avoid carry-out effects), participants came to the laboratory and underwent the steady-state tests after the ingestion of DHC or placebo, in a randomized order. Participants arrived at the same time than on the second visit, and confirmed having met exactly the same pre-experimental conditions. **Fig. 3** shows the design of the steady-state exercise tests.

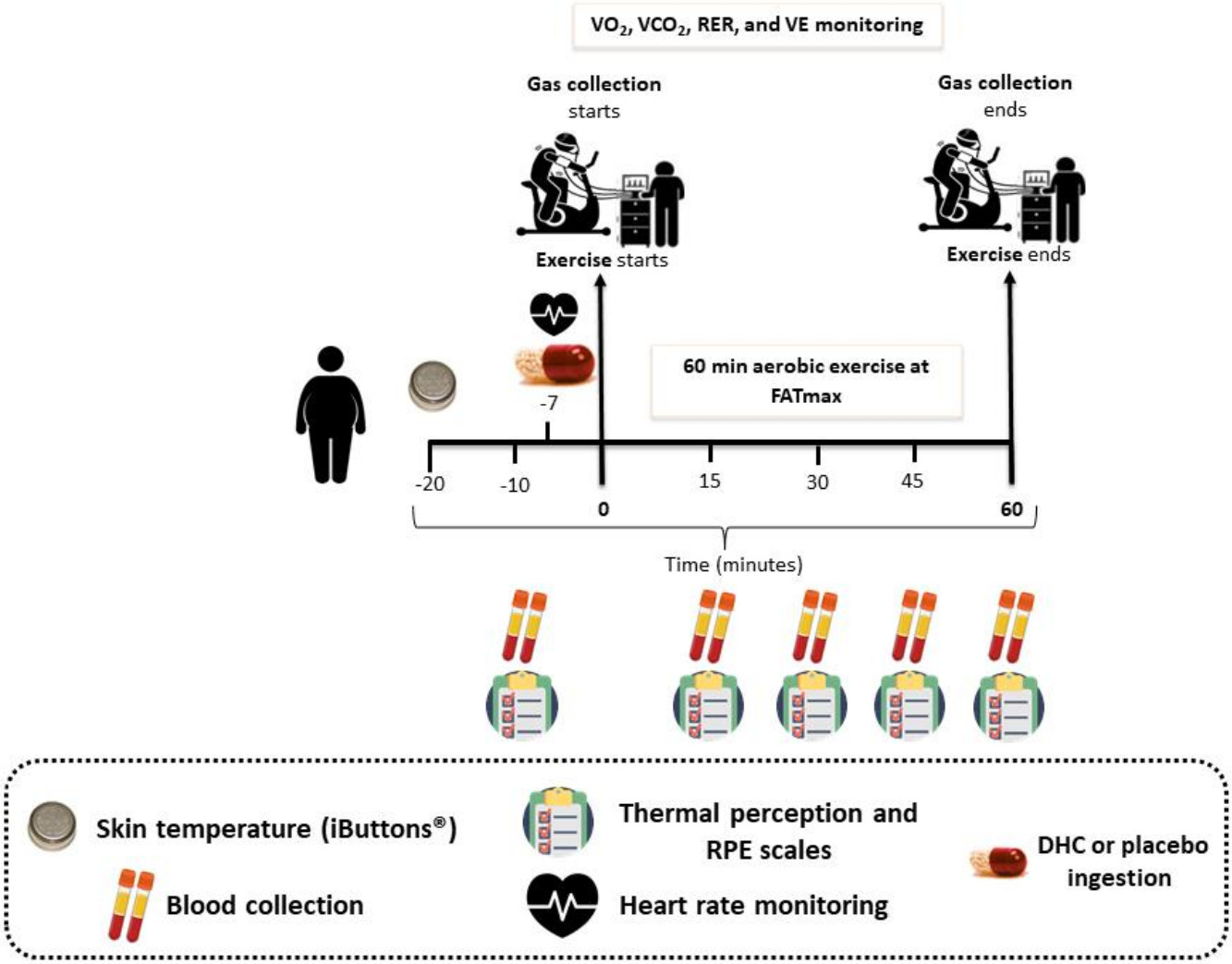

Figure 3. Overview of the steady-state exercise bouts. Abbreviations: DHC: dihydrocapsiate, DXA: dual energy X-ray absorptiometry test, MFO: maximal fat oxidation,VO_2peak: peak oxygen consumption. VE: minute ventilation, RER: respiratory exchange ratio, RPE: rated perceived exertion, VCO_2: volume of carbon dioxide production, VO_2: volume of oxygen consumption.

Participants urinated, dressed in standard clothing, and entered in a quiet, warm(22-23 °C) room. A Polar RS800 heart-rate monitor was placed on their chest using a chest wrap band. Then, a set of 16 DS-1922 L iButton™ wireless thermometers (Thermochron, Dallas, TX, USA) were attached to the subject's skin in different places to monitor skin temperature changes through the experiment. They were put on the forehead, left pectoralis, left elbow region, left index fingertip, left forearm, rear neck central area, right clavicula, right deltoid, right shinbone, right sub-clavicular area, right supra-clavicular area, right thigh, and upper breastbone. Afterwards, participants sat and stay relaxed for 10 min (resting period, timepoint -20´), time during which they were instructed not to move nor cross their arms and legs, and their baseline skin temperature and heart rate measures were taken.

The first intravenous blood sample was collected 10 min before starting the steady-state test (timepoint -10'). Immediately 3 min after the first blood collection (timepoint -7'), participants ingested either 12 mg of DHC (4 pills 3 mg each one) or placebo. Then they sat in the cycloergometer where the steady-state tests would be performed, and they put on their faces a gas mask for the gases exchange measurement. The same metabolic cart than on the second visit was used. The gas collection started 1 min before the beginning of the steady-state test (timepoint -1') with the participants sat in the cycle ergometer without pedaling. After 1 min of gases recording in resting conditions, the steady-state test at FATmax intensity started and continued (constant intensity) until the minute 60, moment at which the test finished. Gases exchange and heart rate were continuously monitored. At time points 15, 30, 45 and 60 min, blood samples were collected to determine serum levels of glucose, triglycerides, and non-esterified fatty acids (NEFAs). Simultaneously, participants completed the ASHRAE scale to record their thermal perception. Every 5 min, participants were asked to report their fatigue perception using RPE scales.

Test substances: dihydrocapsiate and placebo

We employed Capsiate Gold™ soft-gel capsules from Ajinomoto® (Ajinomoto Health & Nutrition North America, Inc, JP) These capsules consisted of 3 mg of purified DHC vehiculated with canola oil, modified corn starch, vegetable glycerin, carrageenan, water, disodium hydrogen phosphate and soy lecithin. Microcrystalline cellulose powder (Fagron Ibérica, Terrassa, SP) was used as placebo. Both DHC and hemicellulose were encapsulated by independent manufacturers, and put in different containers by an independent researcher (not involved in the current study). Each container was labelled with a different code (0 or 1) and, thus, evaluators were not aware of the administered substance – therefore preventing bias. Of note, both DHC and placebo capsules looked

exactly similar to unable the identification of the content by either researchers or participants.

Outcomes

MFO, FATmax and cardiorespiratory fitness

Gas exchange data were obtained and exported from the metabolic carts Breeze Suite (8.1.0.54 SP7) software (MGC Diagnostics Corp.) to Excel for Windows. During the submaximal exercise test, VO_2 and VCO_2 data were averaged over the last 60 s of each 3 min stage [22], and FATox was estimated from these values by using Frayn stoichiometric equations [23] (shown below; urinary nitrogen excretion was assumed to be negligible) [24]. The obtained FATox values (g/min) from the different stages of the submaximal exercise test were plotted against the relative exercise intensity (W). A third-degree polynomial regression was subsequently built to determine the absolute maximal fat oxidation (MFO, g/min). FATmax was calculated as a function of VO_2peak (i.e., % VO_2peak) by selecting the VO_2 value at the temporal moment at which MFO was elicited, and plotting this value against the estimated VO_2peak, expressing it as a percentage. Maximal VO_2 (VO_2max) was defined as a respiratory exchange ratio of ≥1.1, once a VO_2 plateau was reached and having attained a heart rate values within 10 beats/min of the individuals' age-predicted maximum (209-0.73×age) [25] during the maximal exercise test. However, participants did not achieve the VO_2max criteria, and therefore VO_2 peak was determined as the highest VO_2 value that was not an artifact. This value was provided relative to body mass.

Gases exchange parameters during steady-state tests

Gas exchange data were downloaded and averaged every 1 min as above stated. Then, VO_2 and VCO_2 for each selected data point were used to estimate EE, RER, and nutrient oxidation rates – comprised of carbohydrate oxidation (CHOox) and FATox. EE was estimated using Weir's abbreviated equation [26]. Frayn's stoichiometric equations [23] were used for estimating the CHOox and FATox. Urinary nitrogen excretion was assumed to be negligible, and therefore was not included in the formula [27].

$$\textit{Energy expenditure (Kcal/min)} = (1.106 * VCO_2) + (3.941 * VO_2)$$

$$\textit{RER} = (VCO_2 / VO_2)$$

$$\textit{CHOox (g/min)} = (4.55 * VCO_2) - (3.21 * VO_2)$$

$$\textit{FATox (g/min)} = (1.67 * VO_2) - 1.67 * VCO_2)$$

To calculate the EE variables that were used in the analyses, the 60 min-duration steady stage was split into 5 min stages, and the average EE of each stage calculated. Therefore, a total of 12 mean EE values (one per each stage) were obtained. These values were used for the analyses examining the kinetics of EE. Next, the area under the curve (AUC, trapezoidal rule) and the AUC expressing it as a percentage of its baseline - AUC (% baseline) - were calculated. The same procedure was followed to calculate the RER, CHOox, and FATox during the steady state test. The obtained parameters were used in the subsequent analyses.

Serum parameters

Serum glucose, NEFAs, total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), triglycerides (TG), and liver enzymes (alkaline phosphatase [ALP], gamma-glutamyl-transferase [GGT], and glutamate-pyruvate transaminase [GPT]) were assessed following standard methods using an AU5832 automated analyzer (Beckman Coulter Inc., Brea, CA, USA). Low-density lipoprotein-cholesterol (LDL-C) was estimated as [TC – HDL-C – (TG/5)], with all units expressed in mg/dL [28]. Serum insulin was measured using the Access Ultrasensitive Insulin chemiluminescent immunoassay kit (Beckman Coulter Inc., Brea, CA, USA). The homeostatic model assessment for insulin resistance index (HOMA-Index) was calculated as [insulin (μU/mL) × glucose (mmol/L)/22.5 [29]], whereas fatty liver index was calculated using a commonly used procedure [30]. C-reactive protein was measured by an immunoturbidimetric assay using an AU5832 automated analyzer (Beckman Coulter Inc., Brea, CA, USA).

Skin temperature

A total of 16 iButtons® were attached to the skin in different spots (as previously explained). Skin temperature measurements during the steady-state were taken every 60 s using DS-1922 L Thermochron iButtons® (resolution: 0.0625 °C) (Maxim, Dallas, USA) [31]. The iButtons® programming, as well as the downloading and pre-processing of raw data were conducted using Temperatus® software [32]. After conducting the experiment, data were downloaded every 60s for each iButton, in an csv file. Atypical data were eliminated by suppressing the time points for which the rate of change with respect to the previous value was higher than the interquartile distance between quartiles 1 and 3 for all data (percentiles 25 and 75, respectively; [33]). Then, these data were divided in blocks of 5 min, and their average was calculated, obtaining 12 mean values (one for each 5 min-block of the 60 min steady-state test). Finally, the overall mean [34], proximal [35], and distal skin temperatures were calculated using the Temperatus® software - see references [17,36] for further information. The

validity and reliability of this system have been established for the assessment of skin temperature in humans [33,37]. The equations used (see below) have been described elsewhere [36].

Overall mean skin temperature = (Forehead*0.07) + (Right Scapula*0.175) + (Left Chest*0.175) + (Right Deltoid*0.07) + (Left Elbow*0.07) + (Left Hand*0.05) + (Right Thigh*0.19) + (Right Gastrocnemius*0.2).

Proximal skin temperature = (Right Thigh*0.383) + (Right Clavicular*0.293) + (Right Abdomen*0.324).

Distal skin temperature = (Left Hand+Right Instep)/2

Thermal perception

Thermal perception was assessed using the scale from the American Society of Heating, Refrigerating and Air Conditioning Engineers (ASHRAE), which is composed of 7 items in which subjects are asked about their thermal perception over the whole body and different body regions (the clavicular and abdominal regions, arms, hands, legs, and feet). Each scale´s item ranges from cold (-3), cool (-2), slightly cool (-1), neutral (0), slightly warm (1), warm (2), to hot (3). Shivering perception was measured using a numerical scale which ranges from 0 to 10, where 0 means "I am not shivering" and 10 means "I am shivering a lot".

Sample size

Based on previous studies [38], a total of 12 participants per group would be needed to be able of establishing statistical differences between conditions (placebo vs. DHC) in EE (~10%) and FATox (~10-15%) in resting conditions (80% statistical power; α=0.05). Since this is a cross-over study - each participant serves as its own control - the minimum of participants required is n=12.

Randomization

Participants ingested DHC or placebo prior to the steady-state test in a randomized order. This randomization was performed with Excel's Data Randomizer Function (without blocking for any variable or imposing restrictions) by FJAG – who did not participate in the assessments nor experiments.

Blinding

The triple-blinding design consisted of: i) an independent third researcher (not directly involved in this study) conducted the encapsulation of both DHC and placebo in standard pills to unable the identification of the substance by either participants or researchers. These pills were named condition 1 or condition 2

pills; ii) none of the researchers involved in the experimental phases and assessments knew whether the condition 1 or 2 corresponded to DHC or placebo, neither did the participants; iii) during the data and statistical analyses, no one of the team members were aware of the content of conditions 1 and 2, except FAM, who was the coordinator of data analysis (i.e. data analysis was also blinded); v) only when all the statistical analyses were conducted and finalized by FJOP, the content of condition 1 and 2 pills was unveiled.

Statistical analysis

Descriptive statistics of the study subjects are shown as mean ± standard deviation. Data normality was assessed using the Shapiro-Wilk test, histograms, and Q-Q plots. The parameters that did not follow a normal distribution (i.e., gases parameters) were log10-transformed to achieve normal data distribution. Gases exchange parameters during aerobic exercise in the DHC vs. placebo condition were compared using T-test for paired samples. Linear mixed model analyses were used to examine the kinetics of gases exchange parameters during exercise after DHC or placebo ingestion. These analyses (i.e., paired T-tests and linear mixed models) were replicated with the serum analytes, skin temperature and thermal perception across conditions. The level of significance was set at $P<0.05$. The statistical analyses were performed using the Statistical Package for the Social Sciences v.26.0 (IBM Corporation, Chicago, IL, USA). GraphPad Prism version 8.0.0 (GraphPad Software, San Diego, CA) was used to plot the figures including respiratory exchange and blood parameters.

RESULTS

As shown in the **Fig. 1**, from the 32 subjects who were recruited from October 2019 to February 2020 and completed the basal assessment and fulfilled the conditions after the medical check (i.e., they met the inclusion criteria and were therefore enrolled), 5 of them refused to continue because of problems with adhering to the time schedule and experimental conditions. Other 3 subjects were excluded after being randomized and undergoing the steady-state test, due to problems or abnormalities during these tests. Therefore, a final sample size was included for our main analyses (n=24), having all participants valid and complete gases exchange data collection. The sample size varied for the measures related to the secondary aim. The characteristics of the participants are shown in **Table 1.**

Table 1. Descriptive data of the study subjects (n=24).

	N	Mean		SD
Age (years)	24	40		9
Anthropometry and body composition				
Body mass index (kg/m²)	24	31.6	±	4.5
Waist circumference (cm)	24	107.1	±	11.1
Lean body mass (kg)	24	58.1	±	6.6
Lean mass index (kg/m²)	24	19.0	±	2.3
Fat mass (kg)	24	33.7	±	8.6
Fat mass index (kg/m²)	24	11.0	±	2.7
Body fat percentage (%)	24	35.3	±	4.9
Visceral adipose tissue mass (g)	24	818	±	320
Fasting cardiometabolic profile				
Glucose (mg/dL)	23	94	±	8
Insulin (µIU/mL)	23	11	±	6
HOMA-index	22	2.6	±	1.4
GTP (IU/L)	23	33	±	15
GGT (IU/L)	23	42	±	29
ALP (IU/L)	23	72	±	21
Total cholesterol (mg/dL)	23	201	±	28
HDL-C (mg/dL)	22	48	±	9
LDL-C (mg/dL)	22	130	±	21
Triglycerides (mg/dL)	23	129	±	51
Systolic blood pressure (mmHg)	24	126	±	14
Diastolic blood pressure (mmHg)	24	86	±	11
MFO, FATmax and cardiorespiratory fitness				
MFO (g/min)	24	0.24	±	0.09
MFO/LM (mg/kg/min)	24	4.05	±	1.43
FATmax (%VO_2peak)	24	33	±	7
VO_2peak (mL/min)	24	2845	±	473
VO_2peak/lean mass (mL/kg/min)	24	30	±	6

Data are presented as mean and standard deviation (SD). ALP, alanine phosphatase, GGT: gamma-glutamyl transferase, GPT: glutamate-pyruvate transaminase, HDL-C: High density lipoprotein-cholesterol, HOMA: homeostatic model assessment, LDL-C: Low density lipoprotein-cholesterol, LM: lean mass, MFO: maximal fat oxidation, VO_2: volume of oxygen.

Dihydrocapsiate ingestion does not increase energy expenditure or fat oxidation during aerobic exercise at FATmax intensity

No differences across conditions were found in the AUC (% baseline) of EE, FATox and CHOox during exercise **(Fig. 4; Panels A-C)** (All P≥0.219).

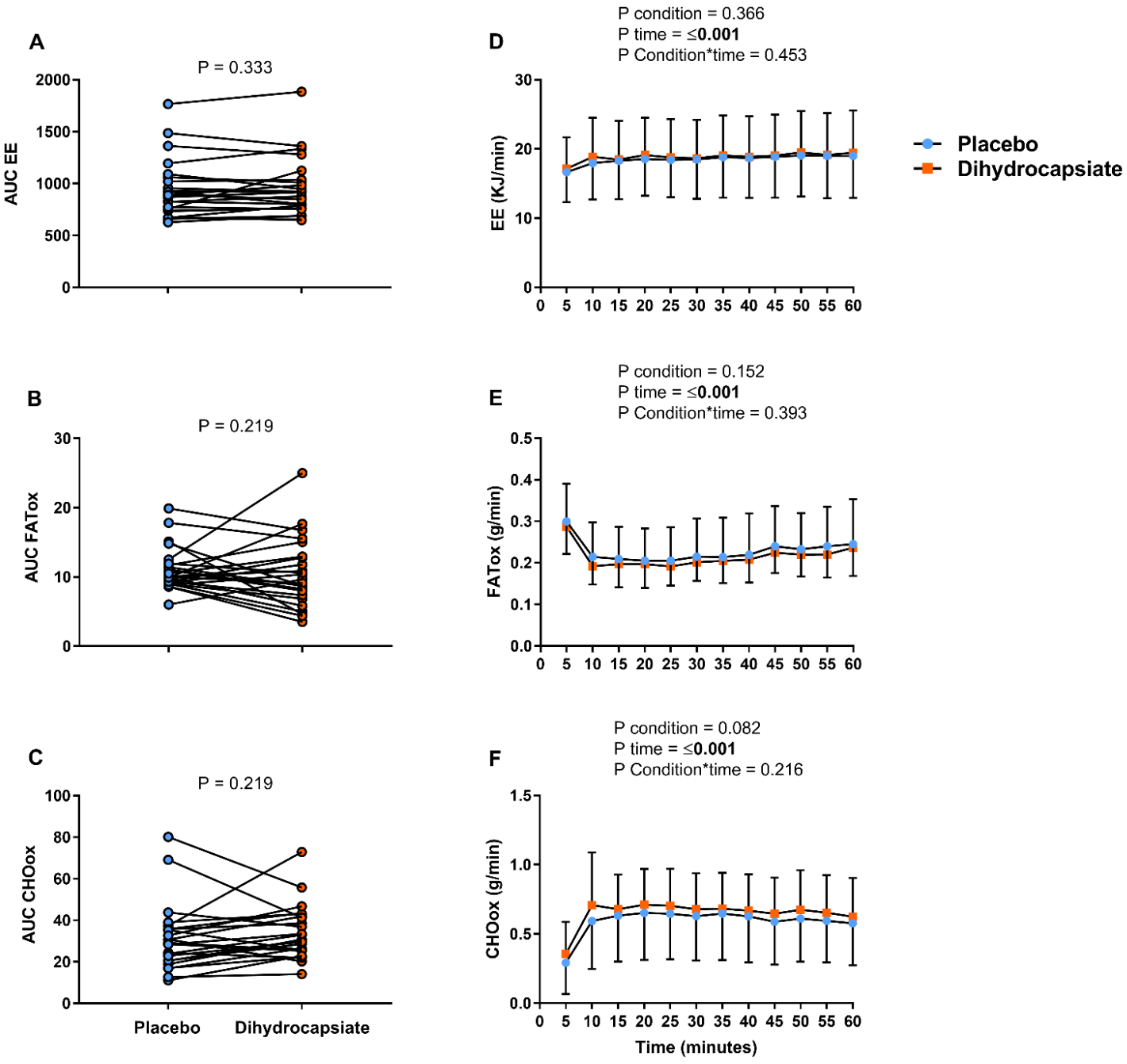

Figure 4. Effects of dihydrocapsiate ingestion on EE, FATox and CHOox during aerobic exercise at FATmax intensity in men with overweight/obesity (n=24). **Panels A, B,** and **C** show the total AUC (an indicator of the overall change) of the EE, FATox and CHOox in the placebo vs. dihydrocapsiate conditions; P values from paired t-test comparing AUC expressed as a percentage of its baseline. **Panels D, E,** and **F** show the kinetics of EE, FATox and CHOox across these conditions; P values from linear mixed model analyses. AUC: area under the curve, CHOox: carbohydrate oxidation, EE: energy expenditure, FATox: fat oxidation. In panels D, E and F, each single point (blue) or square (orange) represents the mean value of each 5 min period.

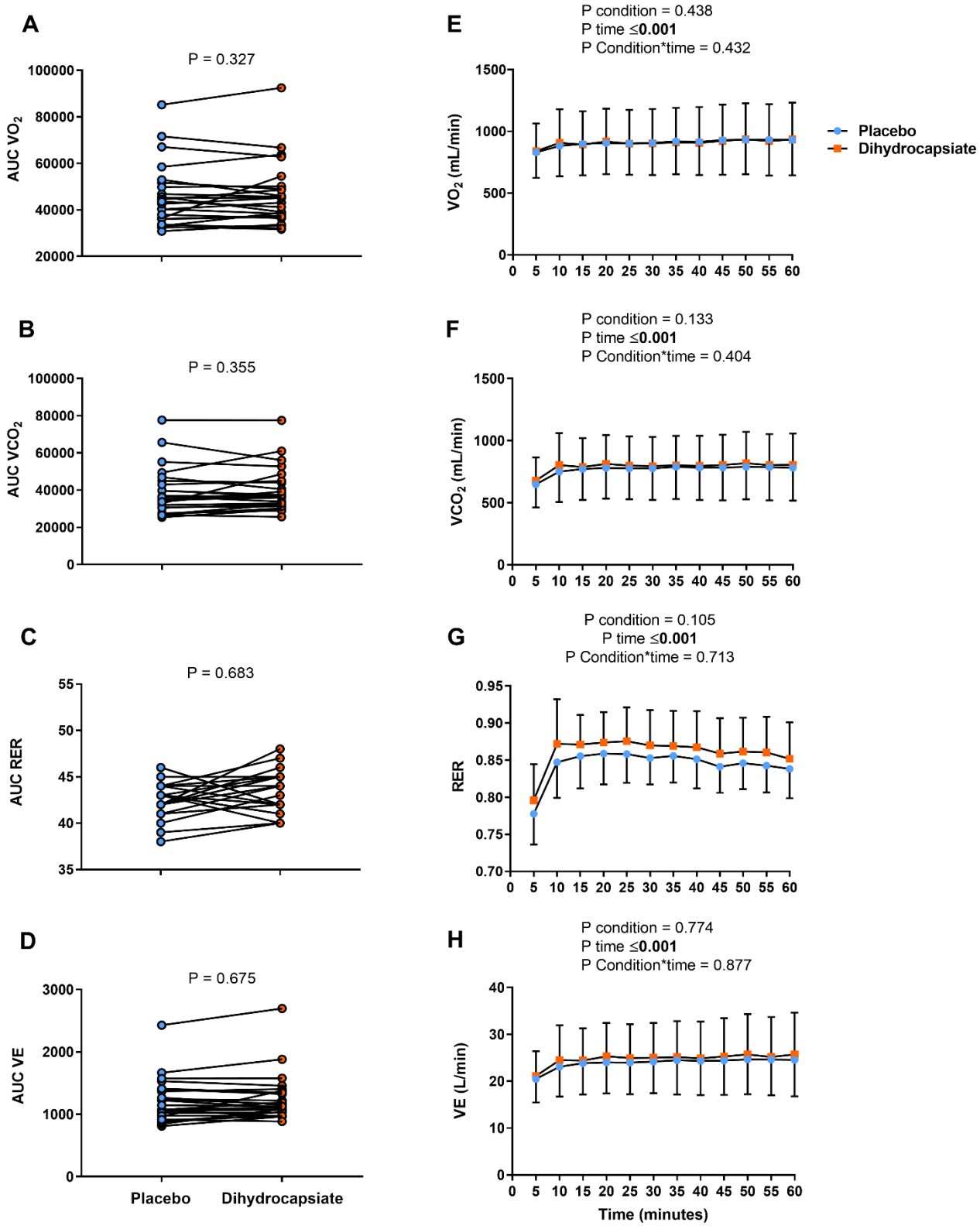

Figure 5. Effects of dihydrocapsiate on VO_2, VCO_2, RER, and VE during exercise in men with overweight/obesity (n=24). **Panels A, B, C** and **D** show the total AUC of VO_2, VCO_2, RER, and VE in the placebo vs dihydrocapsiate conditions; P values from paired t-test comparing AUC expressed as a percentage of its baseline. **Panels E, F, G** and **H** show the kinetics of VO_2, VCO_2, RER, and VE across these conditions. AUC: area under the curve, VE: minute ventilation, RER: respiratory exchange ratio, VCO_2: volume of carbon dioxide production VO_2: volume of oxygen consumption.

Additionally, when the AUC (% baseline) of more raw estimates (i.e., VO_2, CO_2, RER, or the minute ventilation (VE)) during exercise was examined, no differences were either observed across conditions **(Figure 5; Panels A-D)** (All P≥0.327). The kinetics of gases exchanges parameters showed that their values rapidly changed at the beginning (first 5 min) of the steady state (effect of time, P<0.001; **Fig. 4, Panels D-F; Fig. 5, Panels E-H**) but they remained stable from there on. Neither the condition nor the interaction condition*time had a significant effect on EE, FATox and CHOox (**Fig. 4; Panels D-F)** (All P≥0.216) nor on VO_2, CO_2, RER and VE (**Fig. 5, Panels E-H)** (All P>0.404). Results also revealed that the ingestion of DHC had no effect on heart rate (**Fig 6, Panels A-B**) or perceived fatigue (data not shown), confirming that all participants underwent the exercise test under steady state conditions.

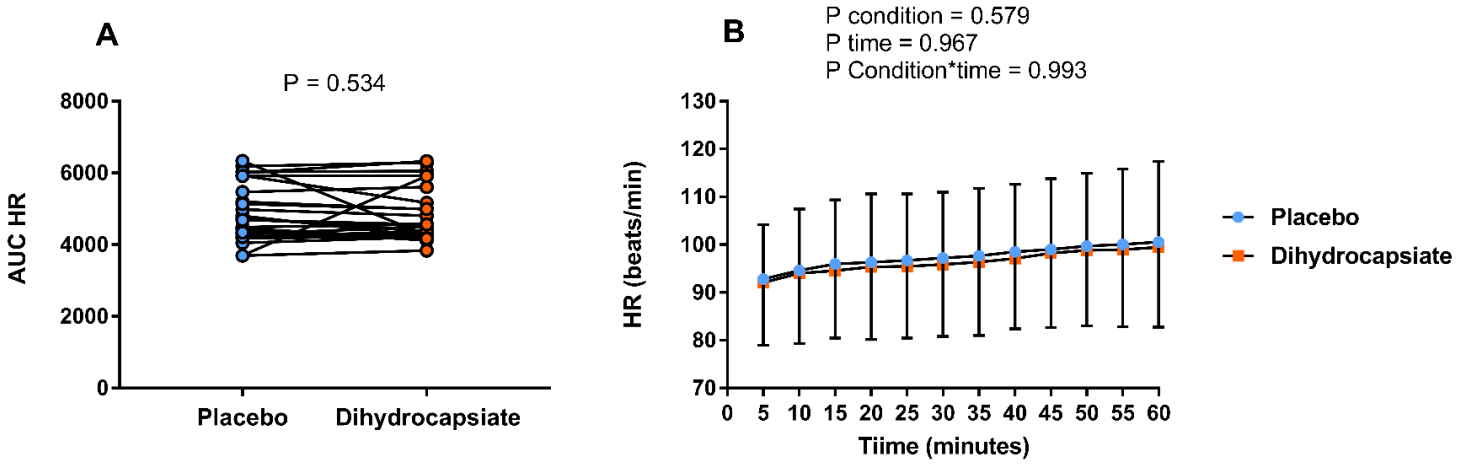

Figure 6. Effects of dihydrocapsiate on heart rate in men with overweight/obesity (n=24). **Panel A** shows the total AUC of heart rate in placebo vs. dihydrocapsiate conditions; P value from paired t-test comparing AUC expressed as a percentage of its baseline. **Panel B** shows the kinetics of heart rate across these conditions. AUC: area under the curve.

Dihydrocapsiate ingestion does not affect blood parameters, skin temperature or thermal percept ion during aerobic exercise at FATmax

The ingestion of DHC had no effect on serum levels of glucose, triglycerides or NEFA kinetics during exercise (**Fig 7, Panels A-C)** (All P≥0.192). Further, the kinetics of mean, proximal and distal skin temperatures during exercise were similar between DHC and placebo conditions (**Fig 8, Panels A-C)** (All P≥0.328), as was the case for the thermal perception of the participants in the whole body, hands, feet and abdominal areas (**Fig. 9, Panels A-D**) (All P≥0.724).

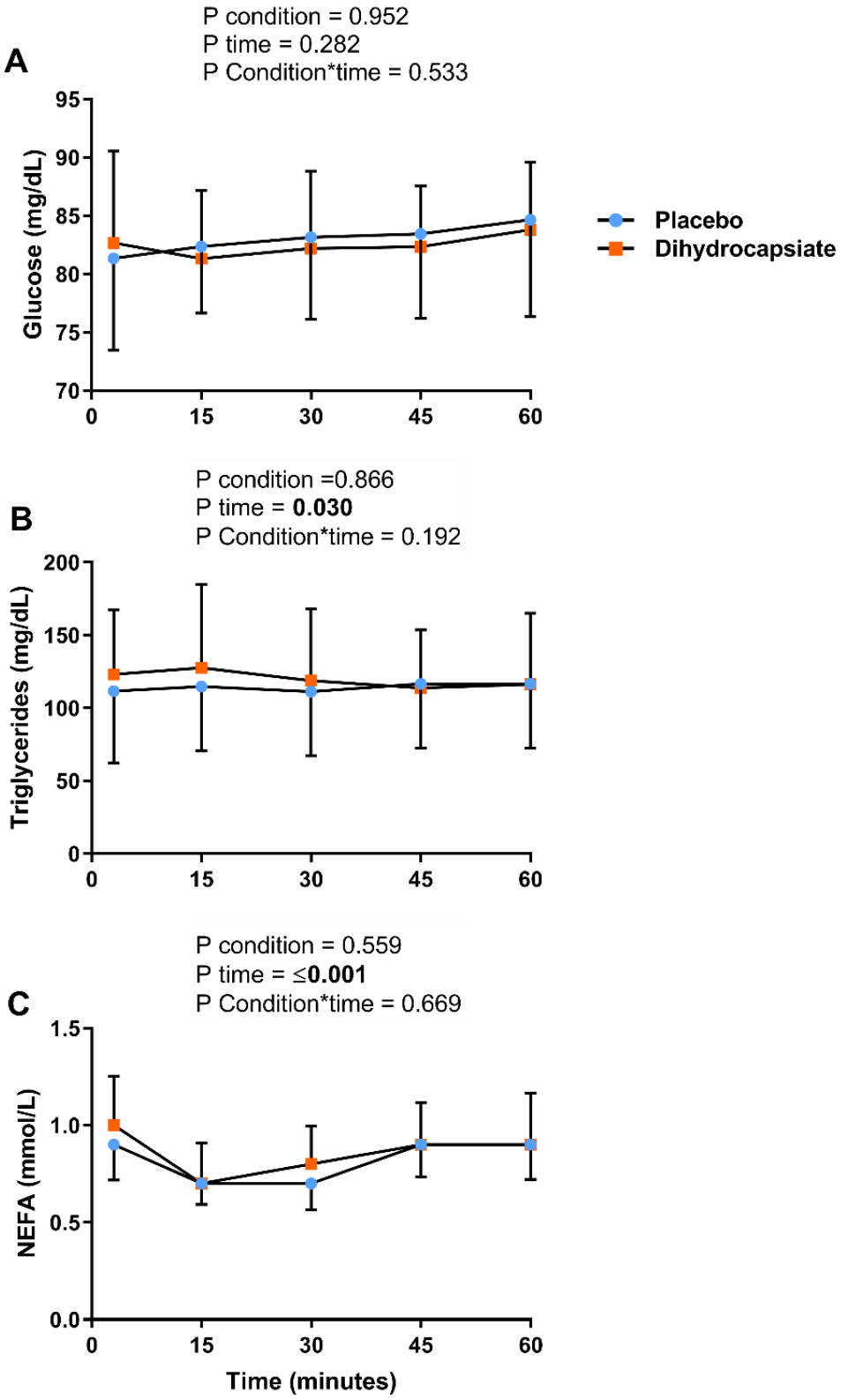

Figure 7. Effects of dihydrocapsiate ingestion on blood parameters during aerobic exercise at FATmax intensity in men with overweight/obesity. **Panels A, B** and **C** respectively show the kinetics of the serum levels of glucose (n=22), triglycerides (n=22), and NEFA (n=16) during exercise in the placebo vs. dihydrocapsiate condition. NEFA: non-esterified fatty acids. P values from linear mixed model analyses.

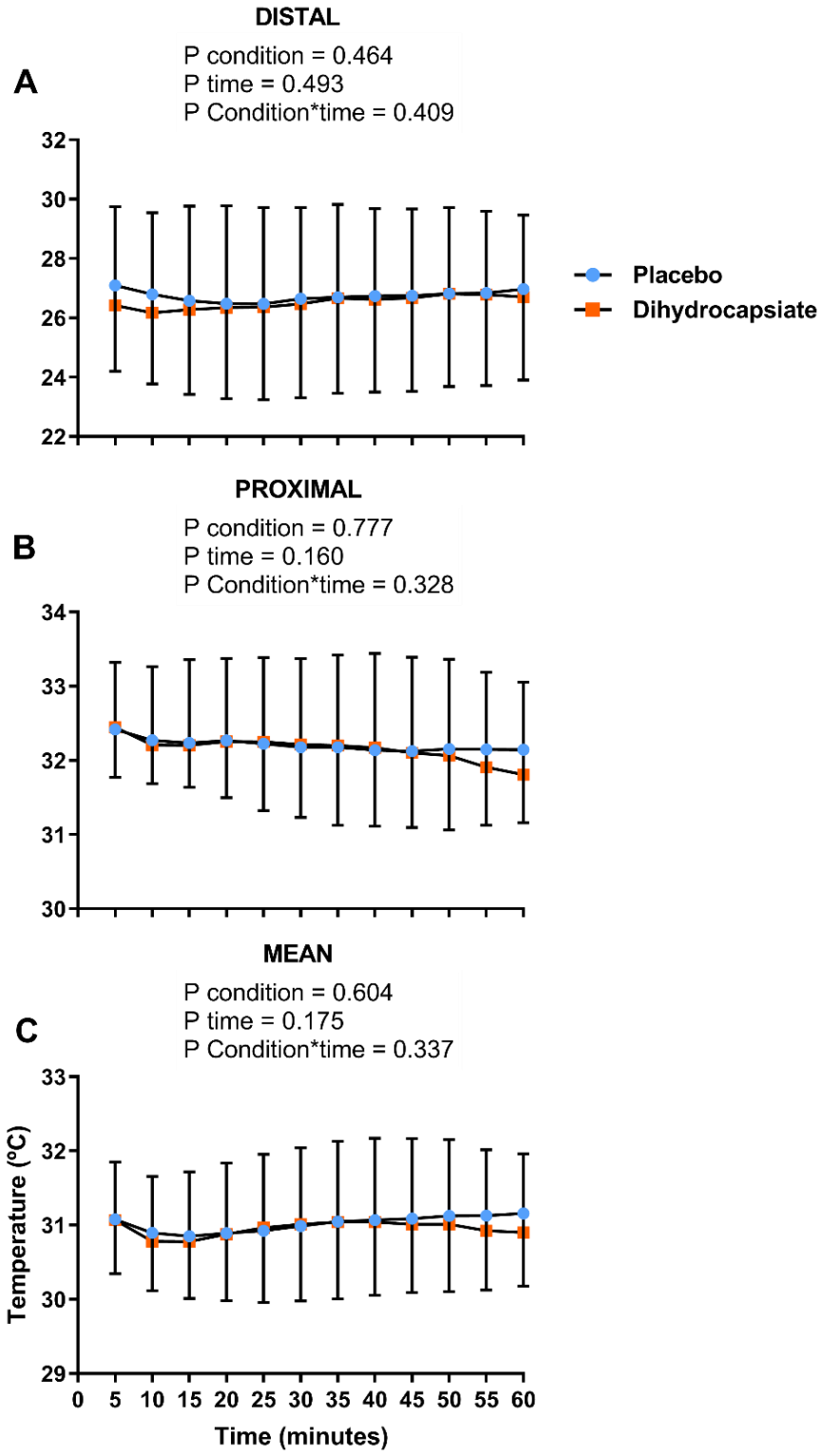

Figure 8. Effects of dihydrocapsiate ingestion on skin temperature during aerobic exercise at FATmax intensity in men with overweight/obesity. **Panels A, B** and **C** respectively show the kinetics of distal (n=22), proximal (n=17), and mean (n=18) skin temperatures during exercise in the placebo vs. dihydrocapsiate condition. Each single point (blue) or square (orange) represents the mean value of each 5 min period. P values from linear mixed model analyses.

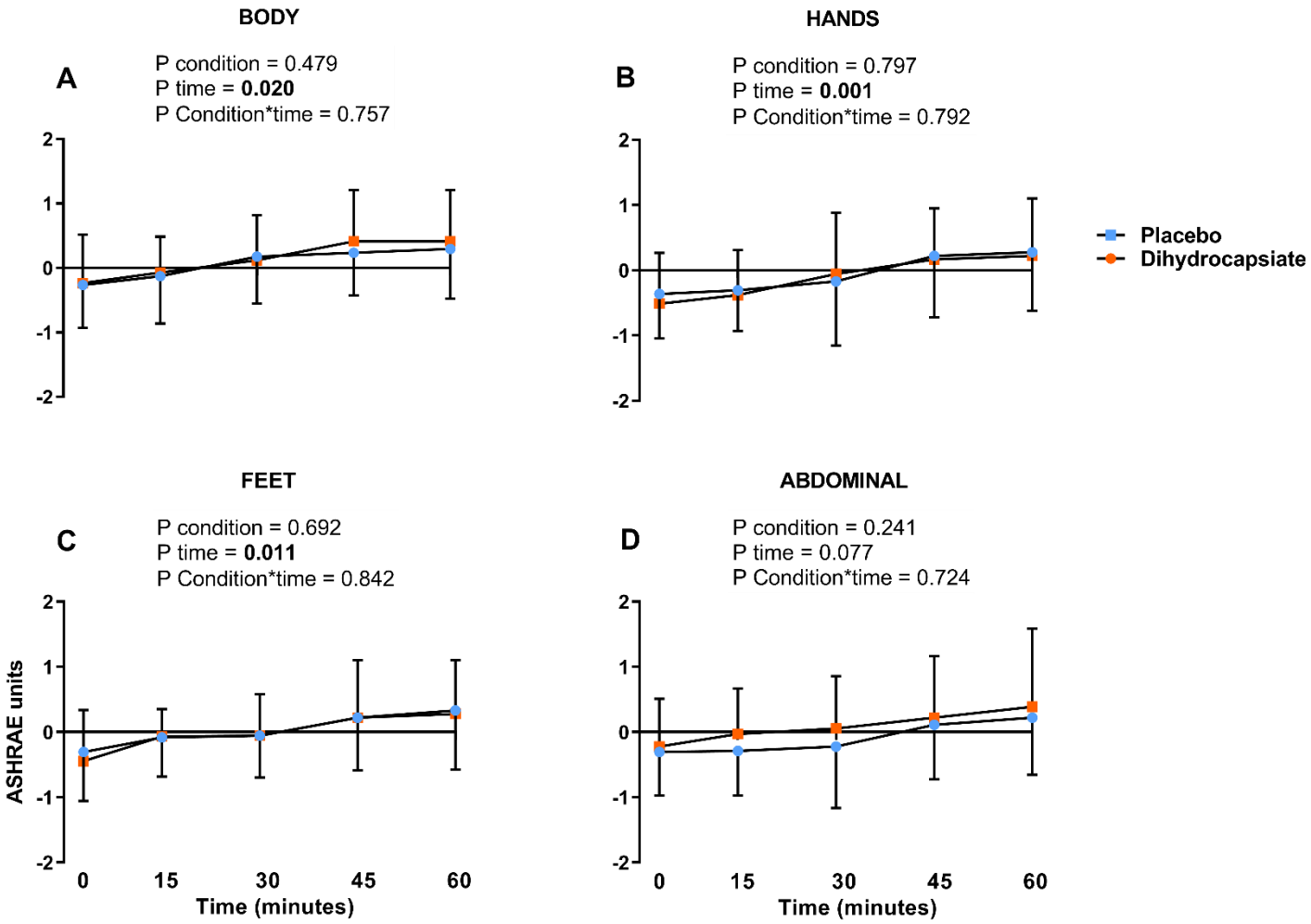

Figure 9. Effects of dihydrocapsiate ingestion on thermal perception during aerobic exercise at FATmax intensity in men with overweight/obesity. **Panel A, B, C** and **D** respectively show the kinetics of thermal perception on body (n=17), hands (n=18), feet (n=18) and abdominal region (n=18). P values from linear mixed model analyses.

DISCUSSION

This study investigated the acute effects of 12 mg of DHC ingestion on EE and FATox during a 60 min steady-state exercise bout at FATmax intensity in men with overweight/obesity. DHC ingestion did not increase EE or FATox as compared with placebo and had no impact on the kinetics of serum levels of glucose, triglycerides, or NEFA. Similarly, DHC had no effect on skin temperature or temperature perception. These findings do not support the use of DHC to increase EE or FATox during aerobic exercise at FATmax intensity in men with overweight/obesity.

Our results concur with those reported by the previous study of Josse et al. [15]. In this study, they tested the ingestion of 10 mg of capsinoids, consisting in a combination of capsiate, DHC, and nordihydrocapsiate (70:23:7 ratio, respectively), in 12 healthy young sedentary and lean men (24.3 ± 3 years old, BMI = 25.5 ± 2 kg/m^2), at rest, during 90 min of cycling at 55% at VO_2 peak, and for 30 min of recovery. Participants ingested capsinoids 30 min before to exercise. Despite the fact they showed that capsinoids increased EE and FATox at rest, there were no significant effects of capsinoids on these parameters during exercise or recovery vs. the placebo condition [15]. Even though there were important differences regarding the participants characteristics and exercise protocols, the findings of Josse AR et al. agree with our results in terms of the absence of effects of capsinoids on EE, FATox, serum NEFA levels, heart rate and perceived fatigue during the exercise.

On the other hand, in a cross-over study by Costa et al., it was demonstrated that the ingestion of 12 mg of capsinoids (capsiate) improved the time-trial performance (400 and 3000 m) in physically active men (28.6±5.4 years old, BMI~24.8) [39]. Participants ingested either 12 mg of capsiate or placebo 45 min before the 400- or 3,000-meter running time trial. The time spent in completing the trial was significantly shorter in those participants who took the capsiate vs. those who took the placebo, but there were no statistical differences in the heart rate or RPE across conditions. Thus, it may happen that capsinoids are not likely to affect EE and FATox during low-intensity exercise activities, but they have an ergogenic role in aerobic exercises that majorly relies on glycolysis as main source of energy, yet the mechanisms are uncertain. However, capsinoids did not improve total time in completing a longer (10,000 m) running time trial [40]. In this study, Von Ah Morano et al. evaluated the effects of 24 mg of capsiate or a placebo in a double-blinded crossover trial in 21 young and lean amateur male athletes (29.3 ± 5.5 years old, BMI~24). Participants ingested 24 mg of capsiate or placebo 45 min before starting the trial, but no differences were found across conditions for the time spent in the tests. Similarly, heart rate or RPE did not differ across conditions [40], which concurs with our results.

Previous evidence has shown that capsinoids intake could enhance resting EE and FATox in humans, particularly in individuals with overweight/obesity [16]. Based on that, we hypothesized that capsinoids intake in combination with aerobic exercise at FATmax intensity would further increase the EE and FATox in men with overweight/obesity. Taken all the results together, it appears that capsinoids are not likely to increase EE nor FATox during low-intensity aerobic exercise, neither in lean nor obese populations – which may be explained because the relative fat oxidation during this lipolytic stimuli is already high (e.g., FATmax) and overshadows any additional contribution of capsinoids. To date,

the clinical potential of capsinoids to maximize the exercise benefits from a metabolic perspective is limited. Nevertheless, the role of capsinoids as ergogenic supplements in glycolytic-dependent aerobic exercise is still to be discerned.

Last but not least, the knowledge about the metabolism and bioavailability of orally ingested capsaicinoids in rodents is well-documented, yet the pharmacokinetics of orally ingested capsinoids is poorly understood [41]. There is solid evidence in humans of capsaicin ingestion increasing performance in aerobic and resistance exercises compared to placebo conditions [4]. Indeed, orally ingested capsaicin is absorbed in the intestine and pass to the bloodstream [42], and thereby it can activates TRPV1 in peripheral muscles [4]. The fact that orally ingested capsaicin can exert its TRPV1 agonism not only within the gut, but also in peripheral tissues, could be explained because capsaicin exerts a greater response than capsinoids in terms of metabolic and ergogenic effects [4]. Contrarily, there is no evidence showing that capsinoids can actually pass into the bloodstream in humans after being orally ingested. Actually, plasma levels of capsinoids and their metabolite, vanillyl alcohol, were below the lower limit of quantitation after ingestion of soft gel capsules containing either 15 or 30 mg of capsinoids [43]. In the light of the current evidence, the mechanisms by which capsinoids could increase EE and FATox - at least when capsinoids are orally ingested in doses below 30 mg- are likely to be explained by TRPV1 activation solely within the gastrointestinal tract [7,44]. Whether the use of higher capsinoids doses or the implementation of systems can increase the bioavailability of capsinoids, and potentially EE and FATox remains to be elucidated.

Strengths and limitations

The main strengths of our study are: i) the study design (i.e., randomized, triple-blinded, placebo-controlled, crossover trial); ii) a well-phenotyped cohort of men with overweight/obesity- a population in which capsinoids could exert a greater effect in terms of increasing EE and FATox [16] - and therefore have a bigger clinical potential; and iii) an accurate monitoring of EE and FATox during exercise, including blood sampling to compare the kinetics of energy substrates; iv) the fact that we employed the highest dose approved by the EFSA in order maximize the response elicited by DHC. However, our study also suffers from limitations: i) no women were included in the trial, so we cannot have any insight about the effects of capsinoids in female participants; and ii) our results cannot be extrapolated to those obtained with different exercise protocols (i.e., having a different exercise type, volume or intensity).

Future research

There are no studies evaluating the effect or aerobic training interventions in combination with capsinoids supplementation on EE and FATox in humans. These studies are needed to unveil whether the chronic ingestion of capsinoids could provide additional positive effects in these variables or other health-related outcomes (e.g., weight loss or cardiometabolic health) in combination with aerobic exercise training. The inclusion of female participants in future studies is also mandatory, since to date no study has evaluated the effects of capsinoids during exercise in women. As estrogen and progesterone strongly influence the physiological responses to exercise, particularly FATox could be highly influenced by sex, and thereby the effects of capsinoids in women' FATox could be significantly different.

CONCLUSION

Our results do not support the use of DHC for increasing EE or FATox during aerobic exercise at FATmax intensity in men with overweight/obesity. Further studies are needed to investigate if these results replicate in other populations and with other exercise types and intensities.

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GENERAL DISCUSSION

INTEGRATIVE DISCUSSION OF THE MAIN FINDINGS

The present International Doctoral Thesis aimed to understand the impact of exercise and bioactive ingredients on novel CMR factors and energy metabolism in adults. The findings from the studies included in the **Section I** revealed that plasma succinate levels might be a promising novel CMR marker in young, sedentary adults, yet succinate levels were not modified after a 24-week of an exercise training program. On the other hand, in **Study II** we demonstrated that acute exercise impacts plasma levels of BA - which also have been proposed as novel CMR markers [1] - and that these levels rapidly decreases after a bout of exercise in an exercise-type specific manner in young, sedentary adults. Notably, individuals with higher CRF levels showed a unique response of primary BA CA and CDCA after EE that seems to be reflective of their better health status in comparison to their low CRF levels counterparts (**Study II**). Overall, the results from **Section II** do not support the ingestion of bioactive ingredients for increasing human BAT volume and/or activity in healthy adults, except for capsinoids (**Study III**). Nonetheless, strong scientific evidence from rodent models supports the use bioactive ingredients to activate BAT and promote WAT browning and thus to potentially combat obesity and cardiometabolic disorders (**Study IV**). Finally, in **Study V** we evaluated the effect of dihydrocapsiate during endurance exercise, concluding that the ingestion of dihydrocapsiate does not increase EE or FATox during aerobic exercise at FATmax intensity in men with overweight/obesity.

Impact of exercise on novel markers of cardiometabolic risk in young adults

The world is witnessing an alarming increase in the incidence of cardiometabolic diseases across young and middle-aged adults [2,3] . This situation calls for the identification and implementation of novel CMR markers for identifying individuals at higher risk of developing cardiometabolic diseases and establishing adequate prevention and treatment strategies [4–6]. Recent advances in omics techniques have led to the identification of novel circulating markers associated with CMR risk, yet only a small fraction have the potential of becoming important diagnostic tools in clinical practice [7,8].

Circulating succinate has aroused as a novel CMR marker in middle-aged adults, accumulating strong evidence that support its implementation in the clinical practice because of its unique metabolic properties [9]. However, whether plasma succinate levels could serve as a novel CMR marker in young individuals has not been addressed. Findings form **Study I** revealed that individuals with higher succinate levels had higher levels of traditional CMR makers, such as visceral adipose tissue (VAT) mass, triglycerides, C-reactive protein, and diastolic blood pressure, and higher levels of novel CMR makers such as pro-

inflammatory omega-6 oxylipins than individuals with lower succinate levels, suggesting that it might be useful as a novel clinical tool to identify young individuals at higher CMR risk. Nowadays, cross-sectional imaging with magnetic resonance image (MRI) or DXA are the reference standard for VAT quantification [10,11], while plasma oxylipins are typically determined by (LC-MS/MS) [11]. None of these techniques are implemented in the clinic as routine assessments because they present a relatively high-cost, require qualified personal to handle the instruments and are time-consuming procedures. In this sense, succinate levels can be easily measured through colorimetric assays and even be included in the automatized blood analytics of clinics and hospital, which could provide a valuable proxy of VAT levels and inflammatory status of the individuals. Indeed, since higher levels of circulating omega-6 oxylipins have shown to be indicative of subclinical inflammation even before observed changes to classic pro-inflammatory cytokines [12], the implementation of the assessment of plasma succinate levels on a daily basis could help to improve the evaluation and stratification of the inflammatory status of individuals.

Previous studies have shown that diet and surgical weight interventions lead to a decrease of plasma succinate levels [13,14]. In the light of these results, we hypothesized that a 24-weeks exercise-intervention program would reduce on plasma succinate levels in a similar fashion. However, plasma succinate levels were not modified after 24-weeks of exercise. These finding findings concur with unpublished data from our group that shows that the levels of traditional CMR markers such as glucose, total cholesterol or LDL-C were neither reduced after 24-weeks exercise in the same cohort of young individuals. In fact, similar studies in in relatively healthy adults have retrieved similar results [15–17]. Hence, we believe that the lack of impact of exercise training on plasma succinate levels might be related to the young age and relatively healthy status of our participants. Nevertheless, several studies have demonstrated that a single bout of endurance or resistance transiently increases circulating succinate levels [18]. Actually, it seems that succinate response to exercise is reflective of an improved glucose metabolism of the individuals, since the peak in circulating succinate measured in the muscle femoral vein during EE was strongly associated with insulin sensitivity post-exercise in young healthy adults [19]. In line with this, the increases in plasma succinate levels were associated with a better glucose and insulin response during a mixed-meal tolerance test (MTT) [13]. Altogether, the integrated information derived from the succinate response to acute physiological stimuli, such as exercise and MTT, seems to be reflective of the cardiometabolic status of the individuals. Future studies are needed to determine the validity of succinate levels as novel CMR marker and the impact of acute exercise on young individuals.

Similar to succinate, several studies have shown that higher levels of circulating BA are linked to an increased risk of obesity and a higher incidence of T2D [20]. Results from our research group has demonstrated, for the first time, that plasma levels of BA are associated with higher levels of CMR makers in young and relatively healthy adults [1]. However, whether exercise can modulate circulating levels of BA remains unexplored. To gain more insights into this topic, in **Study II** we investigated the acute effects of EE and RE on plasma levels of BA in young, sedentary adults. We found that a single bout of exercise rapidly decreases plasma levels of BA in an exercise-type specific manner in young, sedentary adults. Surprisingly, those individuals with higher CRF levels showed a unique increase in plasma levels of the primary BA CA and CDCA 120 min after EE that seems to be reflective of their better health status in comparison to their low CRF levels counterparts. These findings suggest that the response of circulating levels of BA to EE response are linked to the cardiometabolic status of the individuals. Similar to succinate, unpublished data from our group concluded the same 24-weeks exercise-intervention of **Study I** did not modify plasma levels of BA in this population, which strengthens the idea that exercise training interventions do not impact circulating levels of these novel CMR markers in young and relatively healthy populations. The integrative view of the findings of **Study I** and **Study II** highlights the clinical relevance of studying of the acute effects of exercise on circulating levels of novel CMR markers such as succinate and BA in young individuals, as they might be reflective of the cardiometabolic status of the individuals. Finally, exercise training interventions appears not to have an impact on the circulating levels of these CMR markers in young and relatively healthy individuals. Further studies are needed to determine whether the implementation of these type of acute exercise studies may serve to identify individuals at high risk of developing cardiometabolic disease and to evaluate the response of these individuals to therapeutic interventions, such as exercise and diet interventions [21,22].

Given that gut microbiota is a source circulating succinate levels and plays a role in the BA metabolism [23,24], we included fecal microbiota diversity and composition analyses in both **Study I-II**. Nonetheless, we found no association between plasma succinate levels and succinate-producing or succinate-consuming bacteria in **Study I** and neither in the fecal microbiota composition between individuals with low and high CRF levels in **Study II**. It is important to consider that fecal microbiota and composition is highly variable between individuals [25] and thereby, the results from **Studies I-II** should be interpreted with caution. Further research is warranted to unveil whether gut microbiota composition is a contributor of succinate circulating levels and whether it impacts the acute exercise response of circulating BA in humans.

Impact of bioactive ingredients and exercise on energy metabolism in humans

Bioactive ingredients counteract obesity and cardiometabolic diseases by targeting different pathways that lead to increases in energy expenditure and reduction of circulating glucose and/or lipid levels [26]. Hence, the use of bioactive ingredients for the activation of BAT and promotion of WAT browning could yield extra benefits beyond the increase in energy expenditure, constituting a promising tool to treat obesity and cardiometabolic diseases [27].

In **Study III**, we analysed human studies that investigated the effect of both acute and chronic ingestion of bioactive ingredients on BAT activity. Capsinoids, tea extract catechins and ephedrine where the most studied bioactive ingredients. However, capsinoids were the only bioactive ingredient that increased BAT activity in terms of a significant increase of ^{18}F-FDG uptake, yet to a lesser degree than cold exposure [28]. Although it seems plausible to activate human BAT through the ingestion of certain bioactive ingredients in healthy adults, the current level of evidence precludes us from drawing firm conclusions. Remarkably, none of these studies were conducted in individuals with cardiometabolic complications. In this regard, a recent study demonstrated that 1-month of berberine supplementation increased BAT mass and activity, reduced body weight, and improved insulin sensitivity in mildly overweight patients with non-alcoholic fatty liver disease [29]. Thereby, future bioactive ingredients intervention studies should focus on populations with overweight/obesity and cardiometabolic complications. **Study III** also revealed a lack of evidence-based systematic consensus on which bioactive ingredients could be the most effective for increasing BAT activity and/or WAT browning in humans. This fact set up the rationale of **Study IV**, where we analysed rodent studies that evaluated the effects of bioactive ingredients on the activation of BAT and promotion of WAT browning. We found that the most studied bioactive ingredients were tea extract catechins, capsaicin and capsinoids, cacao flavanols, and quercetin. Collectively, the results of **Study III-IV** support the use of capsinoids and tea extract catechins as potential activators of BAT and WAT browning. Moreover, most of the studies included in **Study IV** were conducted in diet—induced obesity models, and a posterior analysis of the effects of bioactive ingredients in CMR markers revealed that the increase in BAT activation and/or WAT browning was accompanied by improvements in cardiometabolic health. Hence, future bioactive ingredients intervention studies should focus on individuals with obesity and cardiometabolic diseases. Further, from a mechanistic point of view, **Study IV** indicated that AMPK and SIRT1 upregulation were linked to the activation of BAT and promotion of WAT browning, which concurs with the finding from previous studies [30,31]. Since both AMPK and SIRT1 are considered therapeutic targets in cardiometabolic diseases [32,33], and given the potential role of BAT in the promotion of cardiometabolic health in humans [34], targeting these pathways

to co-activate BAT and WAT browning by using a synergistic combination of bioactive ingredients is still a promising strategy to combat obesity and cardiometabolic disorders in humans.

Impact of bioactive ingredients and exercise on energy metabolism

Some of the bioactive ingredients identified in **Study III** and **Study IV,** like capsaicin and capsinoids, also seems to enhance exercise performance in humans [35,36]. Among these bioactive ingredients, capsinoids were the only bioactive ingredients identified in **Study III** and **Study IV** that proved to activate BAT and to improve exercise performance in both rodent and human models [35,37,38]. However, the aforementioned studies were conducted exclusively in lean and trained individuals, but not in individuals at risk of developing cardiometabolic diseases, such as sedentary individuals with overweight/obesity [39]. This prompted us to conduct **Study V** to investigate whether dihydrocapsiate ingestion, a type of capsinoid, could further increase energy expenditure and fat oxidation during endurance exercise at FATmax intensity in men with overweight/obesity. Contrary to our results, dihydrocapsiate ingestion did not increase energy expenditure or fat oxidation. However, these results are not extrapolable to other types (i.e., running) and intensities of endurance exercise (higher than FATmax intensity) in which capsiate have demonstrated to improve exercise performance [40]. Future acute and chronic capsinoids studies are needed to confirm whether capsinoids ingestion could enhance the benefits of exercise in individuals with obesity and cardiometabolic diseases.

Interestingly, results from **Study IV** suggest that the mechanism of action of capsinoids involves the activation of a TRPV1 channels in the gastrointestinal tract [41,42]. TRP channels are ubiquitously expressed and they act as transducers for a wide range of both physical and chemical stimuli [43]. In addition to TPRV1, other TRP channels such as TRPA1 and TRPM8 are involved the regulation energy expenditure and body weight regulation [44]. Recent and accumulative evidence suggest that TRP channels are in fact involved in the pathogenesis of obesity and cardiometabolic disorders, and are considered as promising therapeutic targets in the treatment of obesity and cardiometabolic diseases [45]. The existence of various types of TRP also raise the possibility synergistic strategies to activate two or more types of TPR simultaneously. Indeed, a recent study evaluated the anti-obesity potential of a combination capsaicin (TRPV1 agonist), menthol (TRPM8 agonist), and cinnamaldehyde (TRPA1 agonist) for 12-weeks in high-fat diet (HFD)-fed mice [46]. The TRP treatment resulted in activation of BAT and promotion WAT browning and was accompanied the prevention of the increase in body gain together with an improvement in glucose homeostasis

[46]. Similarly, TRP activation through bioactive ingredients could be used in study-designs like **Study V.** Regarding TPR and exercise, TRP agonism has shown to be effective for improving thermal perception [47] and to prevent as cramps during exercise [48]. Thereby, future studies targeting TRP channels through some of the bioactive ingredients identified in **Study IV** are strategies of interest to increase energy expenditure and improve cardiometabolic status and could help to increase the metabolic benefits of exercise and exercise performance.

General limitations

The studies included in this International Doctoral Thesis should present several limitations that should be acknowledged:

- The cross-sectional design of **Study I** precludes the establishment of any cause-effect between plasma succinate levels and the circulating levels of classic and novel CMR markers.

- **Studies I-II** were carried out in young, sedentary, and relatively healthy adults. Thereby, the results from these studies are not extrapolable to older populations (i.e., middle-aged, elderly) or those with obesity or cardiometabolic diseases.

- Fecal microbiota diversity and composition parameters are highly variable between individuals [25]. Therefore, the results from **Studies I-II** should be interpreted with caution

- The BAT assessments of **Study I, Study II** and part of the studies included in **Study III** were performed using ^{18}F-FDG uptake as marker of BAT activity. Although ^{18}F-FDG uptake is the current gold-standard for BAT quantification, it also has limitations in the assessment of BAT metabolic activity and volume [49].

- The differences in the composition of bioactive ingredients, ethnicity, and the lack of control of the seasonality effect of the studies include in **Study III** difficulted its inter-study comparison.

- Similarly, the differences in the composition of bioactive ingredients, dose, and assessments of BAT activity and/or WAT browning difficulted inter-study comparison and interpretation of the results of **Study IV.**

- The systematic search in **Study III** and **Study IV** was as exhaustive and precise as possible; however, due to the heterogenicity of the term "bioactive ingredient" and its synonyms, and the problems related to the heterogenicity in the indexing of these studies in PubMed and Web of Science, it was virtually impossible including *all* the studies analysing the effects of bioactive ingredients on BAT activity and/or WAT browning in rodent and human models.

- **Study V** was conducted in middle-aged adults with overweight/obesity. Whether these results are transferable to populations of different age-range and cardiometabolic status, sex, or whether are replicable upon different exercise type (i.e., running) and intensities (i.e., higher than FATmax) should be addressed in future studies.

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CONCLUSIONS

SPECIFC CONCLUSIONS

The present International Doctoral Thesis aimed to evaluate the impact of exercise on novel CMR markers (**Section I**) and the impact of bioactive compounds and exercise on energy metabolism (**Section II**). The specific conclusions derived from the studies included in each of the abovementioned sections are presented below.

Section 1: Impact of exercise on novel cardiometabolic risk markers

- Plasma succinate levels are linked to a specific pro-inflammatory omega-6 signature pattern and higher visceral adipose tissue levels, and might be useful as a novel clinical tool to identify young individuals at higher cardiometabolic risk. Further prospective studies are needed to confirm its clinical relevance and predictive value as a cardiometabolic risk maker (**Study I**).

- A 24-week supervised exercise training intervention does not modify plasma succinate levels in young, sedentary adults. This could be explained because succinate levels are already in within relatively normal ranges, or because our exercise intervention did not affect the mechanisms that contribute to the elevation of plasma succinate levels (**Study I**).

- A bout of exercise rapidly decreases plasma levels of BA in an exercise-type specific manner in young, sedentary adults (**Study II**).

- Individuals with higher CRF levels showed an increase in plasma levels of the primary BA CA and CDCA above their baseline levels at 120 min after EE. These increases in CA and CDCA after EE seems to be reflective of the better health status of these individuals in comparison to their low CRF levels counterparts (**Study II**).

Section 2: Impact of bioactive ingredients and exercise on energy metabolism

- The current level of evidence does not support the ingestion of bioactive ingredients for increasing human BAT volume and/or activity in healthy adults. Future studies warranted to understand whether bioactive ingredients can activate BAT in humans (**Study III**).

- There is strong scientific evidence supporting the use of bioactive ingredients to activate BAT and promote WAT browning and thus to potentially combat obesity and cardiometabolic disorders in rodents. The most studied bioactive ingredients in rodent are extract catechins,

resveratrol, capsaicin and capsinoids, cacao extract flavanols, and quercetin (**Study IV**).

- The molecular mechanism by which bioactive ingredients activate BAT and promote WAT browning seems to involve the activation of the AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1) pathways (**Study IV**).

- Acute ingestion of 12 mg of does not increase energy expenditure of fat oxidation during endurance exercise at FATmax intensity in men with overweight/obesity and neither impacts the circulating levels of glucose, triglycerides, or non-esterified fatty acids (**Study V**).

GENERAL CONCLUSION

In summary, the present International Doctoral Thesis provides new insights into the impact of acute and long terms effects of exercise on circulating levels of novel CMR markers. Furthermore, the use of bioactive ingredients is a promising strategy to activate brown adipose tissue in individuals with obesity and cardiometabolic diseases, while their beneficial effects during exercise remains to be further explored.

FUTURE PERSPECTIVES

FUTURE PERSPECTIVES

- **Study I:**
    - Prospective studies are needed to confirm the succinate clinical relevance and predictive value as a CMR marker, an whether other type of exercise interventions could have an impact on plasma succinate levels.
    - Future studies should investigate the impact of acute endurance and resistance exercise on plasma succinate levels and evaluate whether they are associated with the metabolic status of the individuals in both healthy and unhealthy individuals.
- **Study II:**
    - The pool of bile acids measured in future studies should be also standardized (i.e., measuring primary, secondary, and both conjugated and unconjugated species of BA) in order to allow comparisons between individuals bile acids and its different gluco- and tauro-conjugated forms.
    - Studies comparing healthy individuals vs. individuals with cardiometabolic complications are warranted to confirm whether the plasma BA levels response to exercise is actually associated with the metabolic status of the individual, and which mechanism are involved in their regulation.
    - Given that plasma bile acids levels seem to be sex-dependent, it remains to be explored the impact of sex on the acute response of plasma bile acids to exercise.
- **Study III:**
    - Future acute and chronic studies evaluating the effects of bioactive ingredients must be conducted in individuals with obesity and cardiometabolic complications.
    - There is a total lack of information on the effect of the bioactive ingredients at the molecular level in BAT and WAT that should be considered in future studies (i.e., subcutaneous and visceral adipose tissue biopsies, brown adipose tissue biopsies).

- **Study IV:**

    - Despite mRNA/protein ratio is thought to be constant [88], it could vary depending on specific tissues and genes. Thus, future studies should include UCP1 protein assessments in their analysis to enable inter-study comparison at UPC1 levels.

    - To gain more insight into the mechanism driving the effects of bioactive ingredients, future studies should include unbiased approaches (e.g., RNA sequencing and proteomics) in the analysis of thermogenic pathways.

- **Study V:**

    - Studies evaluating the effect of aerobic training interventions in combination with capsinoids supplementation on energy expenditure and fat oxidations are warranted.

    - As both estrogen and progesterone levels strongly influence fat oxidation in humans, future studies must be conducted in female individuals to evaluate whether the previous results in men are replicated.

    - Finally, given the wide list of bioactive ingredients with thermogenic and potential ergogenic properties, future studies must consider evaluating the effects of other bioactive ingredients and the explore whether it could exist synergic effects when combining two or more than two in the same extract.

ANNEXES

MANUSCRIPTS DERIVED FORM THE INTERNATIONAL DOCTORAL THESIS

1. **Osuna-Prieto FJ**, Jurado-Fasoli L, Di X, Yang W, Ortiz-Alvarez L, Xu H, Plaza-Florido A, Kohler I, Rensen PCN, Ruiz JR, Martinez-Tellez B. A single bout of aerobic and resistance exercise rapidly decreases plasma levels of bile acids in a different manner in young, sedentary adults: role of physical fitness. *In preparation*

2. **Osuna-Prieto FJ**, Acosta FM, Perez de Arrilucea Le Floc'h UA, Riquelme-Gallego B, Merchan-Ramirez E, Xu H, de la Cruz-Márquez JC, Amaro-Gahete FM, Llamas-Elvira JM, Triviño-Ibáñez EM, Segura-Carretero A, Ruiz JR. *Under Review (Clinical Nutrition)*

3. **Osuna-Prieto FJ**, Rubio-Lopez J, Di X, Yang W, Kohler I, Rensen PCN, Ruiz JR, Martinez-Tellez B. Plasma Levels of Bile Acids Are Related to Cardiometabolic Risk Factors in Young Adults. J Clin Endocrinol Metab. 2022 Feb 17;107(3):715-723. doi: 10.1210/clinem/dgab773. PMID: 34718617; PMCID: PMC8851912.

4. **Osuna-Prieto FJ,** Martinez-Tellez B, Ortiz-Alvarez L, Di X, Jurado-Fasoli L, Xu H, Ceperuelo-Mallafré V, Núñez-Roa C, Kohler I, Segura-Carretero A, García-Lario JV, Gil A, Aguilera CM, Llamas-Elvira JM, Rensen PCN, Vendrell J, Ruiz JR, Fernández-Veledo S. Elevated plasma succinate levels are linked to higher cardiovascular disease risk factors in young adults. *Cardiovasc Diabetol.* 2021 Jul 27;20(1):151. doi: 10.1186/s12933-021-01333-3

5. **Osuna-Prieto FJ**, Martinez-Tellez B, Segura-Carretero A, Ruiz JR. Activation of Brown Adipose Tissue and Promotion of White Adipose Tissue Browning by Plant-based Dietary Components in Rodents: A Systematic Review. *Adv Nutr*. 2021 Dec 1;12(6):2147-2156. doi: 10.1093/advances/nmab084. PMID: 34265040; PMCID: PMC8634450.

6. **Osuna-Prieto FJ**, Martinez-Tellez B, Sanchez-Delgado G, Aguilera CM, Lozano-Sánchez J, Arráez-Román D, Segura-Carretero A, Ruiz JR. Activation of Human Brown Adipose Tissue by Capsinoids, Catechins, Ephedrine, and Other Dietary Components: A Systematic Review. *Adv Nutr*. 2019 Mar 1;10(2):291-302. doi: 10.1093/advances/nmy067. PMID: 30624591; PMCID: PMC6416040

7. Ruiz JR, Martinez-Tellez B, Sanchez-Delgado G, **Osuna-Prieto FJ**, Rensen PCN, Boon MR. Role of Human Brown Fat in Obesity, Metabolism and Cardiovascular Disease: Strategies to Turn Up the Heat. Prog Cardiovasc Dis. 2018 Jul-Aug;61(2):232-245. doi: 10.1016/j.pcad.2018.07.002.

SHORT CURRICULUM VITAE

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Education

2017 – 2022 PhD Student, BIOMEDICINE programm, University of Granada, Spain.

2014-2015 Master degree in NUTRITION AND METABOLISM (Grade: 9.3/10), Faculty of Sciences, University of Córdoba, Spain

2010-2014 bachelor's degree in BIOLOGY (Grade: 9.13/10), Faculty of Sciences, University of Granada, Spain.

National Fellowships

2013-2014 Training grant for the students of the last year of bachelor studies. Spanish Ministry of Education. University of Granada, Spain.

2017-2022. FPU research fellow**.** Fellowship for formation of university faculty members from the Spanish Ministry of Education. University of Granada, Spain.

International Fellowships

2019 FPU short-stay international research fellow**.** Leiden Medical University Center (Leiden, The Netherlands). Dept. Medicine Div. Endocrinology Einthoven Laboratory. Professor Patrick CN Rensen. *Duration: 3 months.*

Publications

1. Mendez-Gutierrez A, Aguilera CM**, Osuna-Prieto FJ**, Martinez-Tellez B, Prados MCR, Acosta FM, Llamas-Elvira JM, Ruiz JR, Sanchez-Delgado G. Exercise-induced changes on exerkines that might influence brown adipose tissue metabolism in young sedentary adults. Eur J Sport Sci. 2022 Feb 14:1-53. doi: 10.1080/17461391.2022.2040597. PMID: 35152857.

- JCR IF2020: 4.050; Sport Sciences; Q1, 21/88

2. Osuna-Prieto FJ, Rubio-Lopez J, Di X, Yang W, Kohler I, Rensen PCN, Ruiz JR, Martinez-Tellez B. Plasma Levels of Bile Acids Are Related to Cardiometabolic Risk Factors in Young Adults. J Clin Endocrinol Metab. 2022 Feb 17;107(3):715-723. doi: 10.1210/clinem/dgab773. PMID: 34718617; PMCID: PMC8851912.

- JCR IF2020: 5.799; Endocrinology and Metabolism; Q1, 9/93

3. Osuna-Prieto FJ, Martinez-Tellez B, Ortiz-Alvarez L, Di X, Jurado-Fasoli L, Xu H, Ceperuelo-Mallafré V, Núñez-Roa C, Kohler I, Segura-Carretero A, García-Lario JV, Gil A, Aguilera CM, Llamas-Elvira JM, Rensen PCN, Vendrell J, Ruiz JR, Fernández-Veledo S. Elevated plasma succinate levels are linked to higher cardiovascular disease risk factors in young adults. *Cardiovasc Diabetol.* 2021 Jul 27;20(1):151. doi: 10.1186/s12933-021-01333-3

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4. Osuna-Prieto FJ, Martinez-Tellez B, Segura-Carretero A, Ruiz JR. Activation of Brown Adipose Tissue and Promotion of White Adipose Tissue Browning by Plant-based Dietary Components in Rodents: A Systematic Review. *Adv Nutr*. 2021 Dec 1;12(6):2147-2156. doi: 10.1093/advances/nmab084. PMID: 34265040; PMCID: PMC8634450.

- JCR IF2020: 8.701; Nutrition and Dietetics; Q1, 4/88

5. Osuna-Prieto FJ, Martinez-Tellez B, Sanchez-Delgado G, Aguilera CM, Lozano-Sánchez J, Arráez-Román D, Segura-Carretero A, Ruiz JR. Activation of Human Brown Adipose Tissue by Capsinoids, Catechins, Ephedrine, and Other Dietary Components: A Systematic Review. *Adv Nutr*. 2019 Mar 1;10(2):291-302. doi: 10.1093/advances/nmy067. PMID: 30624591; PMCID: PMC6416040.

- JCR IF2020: 8.701; Nutrition and Dietetics; Q1, 4/88

6. Jurado-Fasoli L, Di X, Kohler I, **Osuna-Prieto FJ**, Hankemeier T, Krekels E, Harms AC, Yang W, Garcia-Lario JV, Fernández-Veledo S, Ruiz JR, Rensen PCN, Martinez-Tellez B. Omega-6 and omega-3 oxylipins as potential markers of cardiometabolic risk in young adults. Obesity (Silver Spring). 2022 Jan;30(1):50-61. doi: 10.1002/oby.23282.

- JCR IF2020: 5.002; Endocrinology & Metabolism; Q2, 40/146

7. Mendez-Gutierrez A, **Osuna-Prieto FJ**, Aguilera CM, Ruiz JR, Sanchez-Delgado G. Endocrine Mechanisms Connecting Exercise to Brown Adipose Tissue Metabolism: a Human Perspective. Curr Diab Rep. 2020 Jul 28;20(9):40. doi: 10.1007/s11892-020-01319-7.

- JCR IF2020: 4.813; Endocrinology & Metabolism; Q2, 46/146

8. Martinez-Tellez B, Sanchez-Delgado G, Alcantara JMA, Acosta FM, Amaro-Gahete FJ, **Osuna-Prieto FJ**, Perez-Bey A, Jimenez-Pavon D, Llamas-Elvira JM, Gil A, Aguilera CM, Rensen PCN, Ruiz JR. Evidence of high 18 F-fluorodeoxyglucose uptake in the subcutaneous adipose tissue of the dorsocervical area in young adults. Exp Physiol. 2019 Feb;104(2):168-173. doi: 10.1113/EP087428.

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- JCR IF2020: 8.194; Cardiac & Cardiovascular Systems; Q1, 31/196

Book chapters

Book chapter in "New Findings from Natural Substances", Bentham Publisher. Chapter title: "New Findings from Natural Substances". *In preparation.*

Scientific and Organising Committee Member Activities

- Organisation staff. II Congreso Nacional / IV Jornadas de Investigadores en Formación: Fomentando la Interdisciplinariedad. 3rd and 4th June, 2019. Granada, Spain.

- Organisation staff. "I Congreso de Investigadores del PTS – Universidad de Granada".

Reviewer activity

FJ Osuna-Prieto has served has reviewer of top indexed journals, such as *Molecular Metabolism, Trends in Food Science & Technology,* and *Metabolism – Clinical and Experimental.*

Participation in Research projects

- **ACTIBATE project:** Activating Brown Adipose Tissue through Exercise. Effects of an exercise intervention on activity and quantity of Brown adipose tissue: A Randomized Controlled Trial.
    - Funded by the Spanish Ministry of Economy and competitiveness among others.
    - Principal investigator: Jonatan R Ruiz.
    - Duration: from 2016 to 2027.
    - Role: data collection and participation on manuscripts.

- **ACTIFOX project**: ACTivating Fat OXidation Through Capsinoids (ACTIFOX). effects of the acute ingestion of dihydrocapsiate during aerobic exercise on energy metabolism in adults with overweigth/obesity.
    - Funded by Consejería de Conocimiento, Investigación y Universidades, Proyectos I+D+i del Programa Operativo del Fondo Europeo de Desarrollo Regional (FEDER 2018, ref. B.CTS.377.UGR18).
    - Principal investigator: Jonatan R Ruiz
    - Duration: from 2019 to 2020.
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Teaching

- Subject**: instrumental techniques of environmental analysis** (60 hours of teaching, 6 credits ECTS).
  Degree/Bachelor: **Environmental Sciences,** University of Granada (Spain). Academic course: **2018/2019.**

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  Degree/Bachelor: **Environmental Sciences,** University of Granada (Spain). Academic course: **2019/2020.**

- Subject: **Analytic chemistry** (60 hours of teaching, 6 credits ECTS).
  Degree/Bachelor: **Pharmaceutics,** University of Granada (Spain). Academic course: **2020/2021.**

Other merits

- 10 **communications** or **posters** both in **national** and **international** conferences.

- **2015.** Award - Best Master Thesis: "*Differential response of adipocytes to prolonged exposure to monounsaturated and saturated fatty acids*". Master's in Nutrition and Metabolism. University of Córdoba. Spain.

- **2021**. **Invited Speaker** at 62 Congreso de la Sociedad Española de Endocrinología y Nutrición (SEEN). Rol del tejido adiposo pardo en la regulación del metabolismo energético y salud cardiovascular. Sevilla, Spain. 13-15th October 2021.

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