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Endocrine function of human brown adipose tissue and its exercise-induced regulators

Función endocrina del tejido adiposo pardo humano y sus factores reguladores inducidos por el ejercicio.

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ABBREVIATIONS

12,13-dihydroxy-9Z-octadecenoic acid (12,13-diHOME)
12-hydroxyeicosapentaenoic acid (12-HEPE)
18F-fluorodeoxyglucose (18F-FDG)
Analyses of covariance (ANCOVA)
Analyses of variance (ANOVA)
Angiopoietin-like protein 4 (ANGPTL4)
Atrial natriuretic peptide (ANP)
World Health Organization (WHO)
Body mass index (BMI)
Bone morphogenic proteins (BMPs)
BP: blood pressure
Brain derived neurotrophic factor (BDNF)
Brown adipose tissue (BAT)
Brown-in-white (brite)
Cardiovascular disease (CVD)
Chemokine C-X-C motif chemokine ligand 14 (CXCL14)
Cyclic adenosine monophosphate (cAMP)
Ependymin-related protein 1 (EPDR1)
Ethylenediamine tetra-acetic (EDTA)
Fibroblast growth factor 2 (FGF2)
Fibroblast growth factor 21 (FGF21)
Fibronectin type III domain containing 5 (FNDC5)
Follistatin-like 1 (Fstl-1)
Growth differentiation factor 15 (GDF15)
Heart rate reserve (HRres)
High-density lipoproteins (HDL)
High-performance liquid chromatography (HPLC)
Homeostasis model assessment of insulin resistance (HOMA-IR)
Hounsfield units (HU)
Interlukin-6 (IL-6)
Interleuquin-4 (IL-4)
LDL: low-density lipoproteins
Maximum oxygen consumption (VO2 max)
MicroRNAs (miRNAs)
Natriuretic peptides (NPs)
Nerve growth factor (NGF)
Type 2 diabetes (T2D)
Neuregulin-4 (Nrg4)
Non-esterified fatty acids (NEFA)
Positron-emission tomography and computed Proliferator-activated receptor gamma coactivator 1-α (PGC-1α)
Proliferator-activated receptor γ (PPARγ)
tomography (PET-CT)
RM: 1 Repetition Maximum
Sympathetic nervous system (SNS)
Standardized uptake value (SUV)
Transforming growth factor-β (TGF-β)
Uncoupling protein-1 (UCP-1)
Vascular Endothelial Growth Factor (VEGF)
VAT: visceral adipose tissue
VO2peak: Maximal oxygen uptake
β-aminoisobutyric acid (BAIBA)

ABSTRACT

Since the presence and activity of brown adipose tissue (BAT) was recognized in human adults in 2009, its study has been gaining interest within the scientific community. Researchers aim to identify non-invasive methods to promote BAT activation as well as to determine its metabolic effects on human health. In addition to the thermogenic activity of BAT, its secretory capacity has been evidenced, and is now recognized that BAT is able to release endocrine signaling molecules, the so-called batokines or brown adipokines. These batokines seem to exert beneficial effects on energy homeostasis and promote white adipose tissue (WAT) browning, a process by which white adipocytes acquire a brown-like phenotype. However, most batokines have been only identified in rodents and *in vitro* models, which is difficult to translate to human physiology. Besides producing endocrine signals, BAT is responsive to many endocrine circulating factors. Studies in mice have suggested that exercise could induce BAT activation and WAT browning, and that this effect may be modulated by the exercise-induced release of endocrine factors, the so-called exerkines. The overall aim of this Doctoral Thesis is to study the endocrine connections of human BAT, by investigating circulating molecules potentially secreted by BAT in response to cold exposure and exercise-induced signals that can regulate BAT metabolism.

The Study I of this Doctoral Thesis attempts to identify the effect of a 2-hour individualized cold exposure on the plasma levels of five potential batokines, previously identified in mice (i.e.: CXLC14, GDF14, FGF21, interleukin-6, and BMP8b). The individualized cooling protocol increased the plasma levels of CXCL14, GDF15, FGF21 and interleukin-6 and decreased the plasma levels of BMP8b. Moreover, the cold-induced changes in circulating FGF21 were positively associated with BAT volume, as measured by a static ^{18}F-FDG PET-CT. These results suggest that human BAT might contribute to the circulating pool of FGF21 upon BAT activation. The Study II aims to characterize the acute and chronic effect of resistance and endurance exercise on the concentration of 16 exerkines known to regulate BAT metabolism. An intense and short bout of endurance exercise elevated plasma levels of noradrenaline, lactate, BDNF, interleukin-6, follistatin-like 1 protein, musclin, and FGF21, whereas it decreased the plasma levels of leptin. Resistance exercise acutely increased lactate levels, but not the other 15 analyzed exerkines. On the other hand, a 24-week training program combining resistance and endurance exercise failed to modulate the circulating levels of these exerkines. Only the endurance acute exercise-induced change in plasma lactate levels were positively associated with BAT parameters, suggesting an inter-organ communication between BAT and skeletal muscle. The findings obtained in this Doctoral Thesis contribute to extend the knowledge on human BAT physiology. These findings may be a starting point for future studies aimed at elucidating the

cardiometabolic effect of BAT activity in humans and the possible development of therapeutic and preventive strategies against obesity and associated diseases.

RESUMEN

Desde que en 2009 se reconoció la presencia y actividad del tejido adiposo marrón (TAP) en adultos humanos, su estudio ha ido ganando interés en la comunidad científica. Los investigadores pretenden identificar métodos no invasivos para promover la activación del TAP, así como determinar los efectos metabólicos que ejerce sobre la salud humana. Además de la actividad termogénica del TAP, se ha evidenciado su capacidad secretora, siendo capaz de liberar moléculas de señalización endocrina, las llamadas batokinas o adipoquinas marrones. Estas adipoquinas marrones parecen ejercer efectos beneficiosos sobre la homeostasis energética y, además, promueven el pardeamiento del tejido adiposo blanco (TAB), un proceso por el cual los adipocitos blancos adquieren un fenotipo similar a los adipocitos marrones. Sin embargo, la mayoría de estas moléculas han sido identificadas solo en roedores y en modelos *in vitro*, lo que resulta difícil de trasladar a la fisiología humana. Además de producir señales endocrinas, el TAP también responde a muchos factores endocrinos circulantes. Estudios en ratones han sugerido que el ejercicio podría inducir la activación del TAP y el pardeamiento del TAB y que este efecto podría estar modulado por la liberación inducida por el ejercicio de factores endocrinos, las denominadas exerquinas. El objetivo general de esta Tesis Doctoral es estudiar las conexiones endocrinas del TAP humano, investigando las moléculas circulantes potencialmente secretadas por el TAP en respuesta a la exposición al frío y las señales inducidas por el ejercicio que pueden regular el metabolismo del TAP.

El Estudio I de esta Tesis Doctoral tiene como objetivo identificar el efecto de una exposición individualizada al frío de 2 horas sobre los niveles plasmáticos de cinco adipoquinas marrones potenciales, previamente identificadas en ratones (CXLC14, GDF14, FGF21, interleucina-6, y BMP8b). El protocolo de frío individualizado aumentó los niveles plasmáticos de CXCL14, GDF15, FGF21 e interleucina-6 y disminuyó los niveles plasmáticos de BMP8b. Además, los cambios inducidos por el frío en el FGF21 circulante se asociaron positivamente con el volumen del TAP, medido mediante un PET-TC estático con ^{18}F-FDG. Estos resultados sugieren que el TAP humano podría contribuir a la reserva circulante de FGF21 tras la activación del tejido. El Estudio II tiene como objetivo caracterizar el efecto agudo y crónico del ejercicio de resistencia sobre la concentración de 16 exerquinas conocidas por regular el metabolismo del TAP. Una sesión intensa y corta de ejercicio de resistencia elevó los niveles plasmáticos de noradrenalina, lactato, BDNF, interleucina-6, proteína 1 similar a la follistatina, musclin y FGF21,

mientras que redujo los niveles plasmáticos de leptina. El ejercicio de resistencia aumentó de forma aguda los niveles de lactato, pero no los de las otras 15 exerquinas analizadas. Por otra parte, un programa de entrenamiento de 24 semanas que combinaba ejercicios de resistencia y aeróbico no consiguió modular los niveles circulantes de estas exerquinas. Sólo el cambio inducido por el ejercicio agudo de resistencia en los niveles de lactato plasmático se asoció positivamente con los parámetros del TAP, lo que sugiere una comunicación endocrina entre el TAP y el músculo esquelético. Los resultados obtenidos en esta Tesis Doctoral contribuyen a ampliar los conocimientos sobre la fisiología del TAP humano. Estos hallazgos pueden ser un punto de partida para futuros estudios dirigidos a dilucidar el efecto cardiometabólico de la actividad del TAP en humanos y el posible desarrollo de estrategias terapéuticas y preventivas contra la obesidad y enfermedades asociadas.

GENERAL INTRODUCTION

1. OBESITY: A GLOBAL EPIDEMIC

Currently, obesity has become one of the chronic disorders that contribute the most to the global burden of disease. It represents a major public health problem worldwide, as its prevalence has increased dramatically in recent years in both children and adults [1]. Indeed, although malnutrition has traditionally been associated more with undernutrition rather than obesity, today the situation has alarming reversed, with the exception of Asia and Sub-Saharan Africa[2]. **Figure 1** show the worldwide prevalence of obesity in adults in 2021.

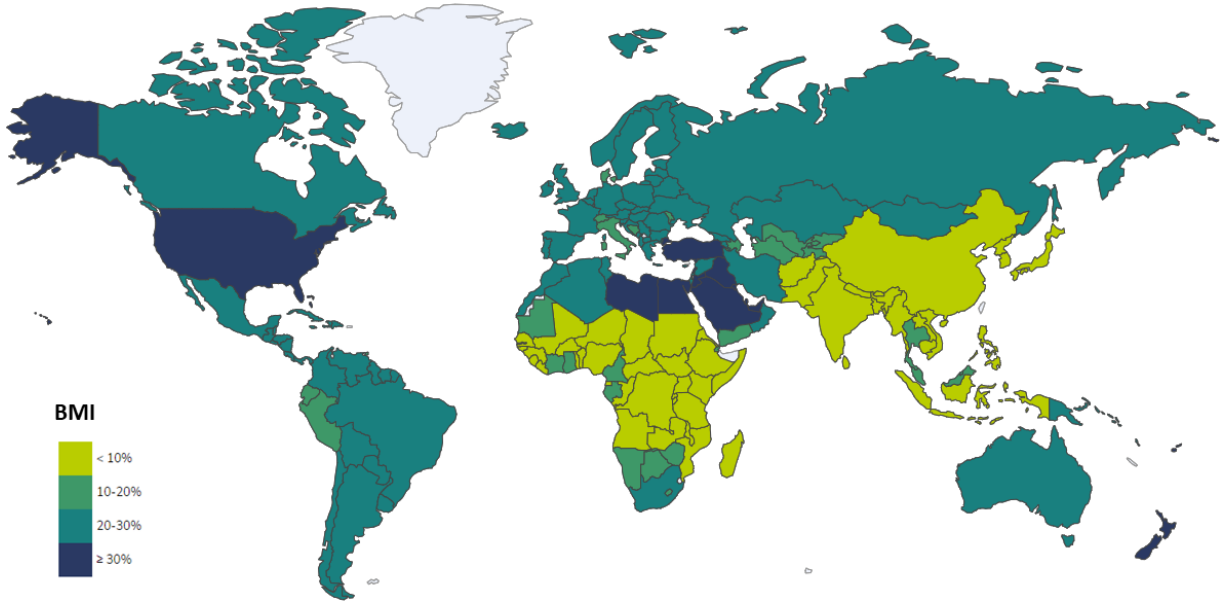


Figure 1. *Worldwide prevalence of obesity in adults (%). Source: Global Health Observatory Data Repository, https://data.worldobesity.org/maps/?area=maps .*

The World Health Organization (WHO) defines overweight and obesity as excessive or abnormal fat accumulation[3]. Body mass index (BMI) is used to determine overweight and obesity, with people being classified as normal weight with BMIs ranging from 18.5 to 24.9, as overweight with a BMI from 25 kg/m^2 to 29.9 kg/m^2 overweight and as having obesity with a BMI ≥ 30 kg/m^2 [3]. Despite this simplistic definition, obesity is a complex and multifactorial disease that occurs as a result of a maintained positive energy balance and a modification of the body weight homeostatically defended by the organism[4]. It is not only determined by diet and physical activity levels, but also by several drivers and determinants including biological, genetic, lifestyle, social and environmental factors[5]. The interactions between genetic profile and environmental factors makes the aetiology of obesity highly complex and, consequently, it is a challenge the discovery of the underlying mechanisms of action.

Prevalence of obesity has alarmingly increased in the last decades, being responsible for approximately 2.8 million deaths in 2021[6]. It is predicted that by 2030, 1 out of each 5 women and 1 out of each 7 men will have obesity, resulting in over 1 billion people worldwide[7]. **Table 1**

shows the estimated global prevalence until 2030, when people living with obesity will almost duplicate in comparison with the rates of 2010.

Table 1. *The estimated global prevalence of obesity until 2030. Modified from NCD Risk Factor Collaboration (2017), UN Population Division and World Obesity Federation projections.*

	2010		2025		2030	
Adults	%	Millions	%	Millions	%	Million
BMI ≥ 30	11.4	511	16.1	892	17.5	1025
BMI ≥ 35	3.2	143	2.4	284	5.7	333
BMI ≥ 40	0.9	42	1.7	93	1.9	111

Obesity is a chronic disease strongly associated with multiple comorbidities, including insulin resistance, cardiovascular disease (CVD), type 2 diabetes (T2D), metabolic syndrome, hypertension, cancer and even mental disorders[8–10], in addition to being the second most predictive factor for coronavirus disease-2019 mortality[11]. Since the wide range of pathologies associated, obesity has several implications not only for people's quality of life and mortality, but also for the economy and the substainability of society. Obesity and its comorbidities represent one of the costliest public health problems facing both developed and developing countries. In fact, it has been estimated that adults with a BMI between 35-40 kg/m^2 or above 40 kg/m^2 cause 63% and 116% higher costs, respectively, than a person of normal weight[12].

The severity and complexity of obesity and associated comorbidities make it urgent and necessary, albeit challenging, to investigate effective therapies for prevention and treatment, improving the quality of life of patients with obesity.

2. BROWN ADIPOSE TISSUE: A PROMISING TARGET FOR OBESITY PREVENTION AND THERAPY

BROWN ADIPOSE TISSUE, AN HISTORICAL OVERVIEW

Brown adipose tissue (BAT) is a thermogenic tissue capable of generating heat when mammals are exposed to temperatures below thermoneutrality[13,14]. Brown adipocytes are characterized by multiple small lipid droplets, a large number of mitochondria and high vascularization[15], which make them to acquire a light pink to dark red tone. A key characteristic of brown adipocytes is the presence of the uncoupling protein-1 (UCP-1) UCP-1 in the inner

membrane of the mitochondria. UCP-1 provides an alternative pathway for H+ molecules to return to the mitochondrial matrix from the intermembrane space. The energy produced through the passage of protons is used to generate heat instead of synthesizing ATP, as would traditionally occur through the action of ATP synthase during cell respiration[16] (**Figure 2**). Due to these characteristics, upon BAT activation, mainly by the sympathetic nervous system (SNS), energy is quickly supplied and dissipated as heat. Oxygen and nutrients (such as fatty acids) necessary for heat dissipation are rapidly supplied by triacylglycerol stores and high vascularity[17].

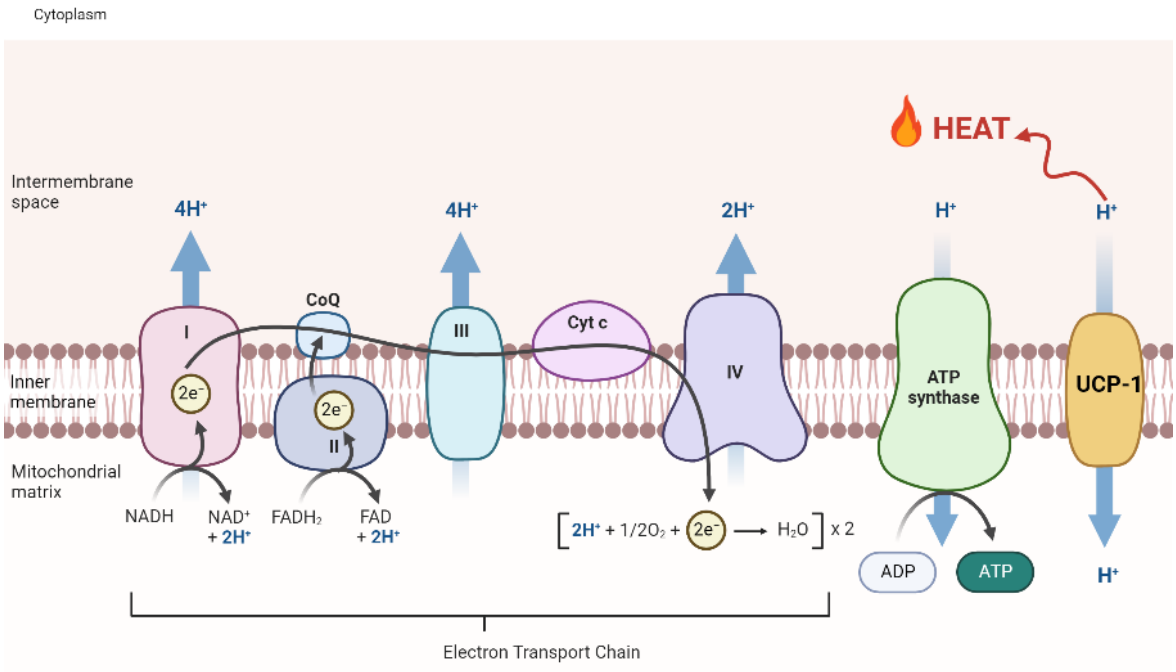


Figure 2 *Heat production by the UCP-1 action in the inner mitochondrial membrane. Adapted from "Electron Transport Chain", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates*

Historically, BAT has been believed to be only present in newborns, being irrelevant in adult humans[18]. Nevertheless, in 2002, with the advent of the radiotracer ^{18}F-fluorodeoxyglucose (^{18}F-FDG) for positron-emission tomography and computed tomography (PET-CT) scanning (used to identify tumors), symmetrical "tumor-like" areas were described in the supraclavicular and neck regions. However, it was not until 2007 that the presence of active and metabolic BAT in adult humans was claimed [19], and finally recognized in 2009[15,20–22]. Consequently, several retrospective studies started to describe the prevalence and localization of BAT depots[15,23,24]. Currently, it is confirmed that BAT exists and has thermogenic activity in human adults[21,25–27].

Human BAT depots distribution is restricted to the neck, shoulders, posterior thorax, and abdomen[28] (**Figure 3**).

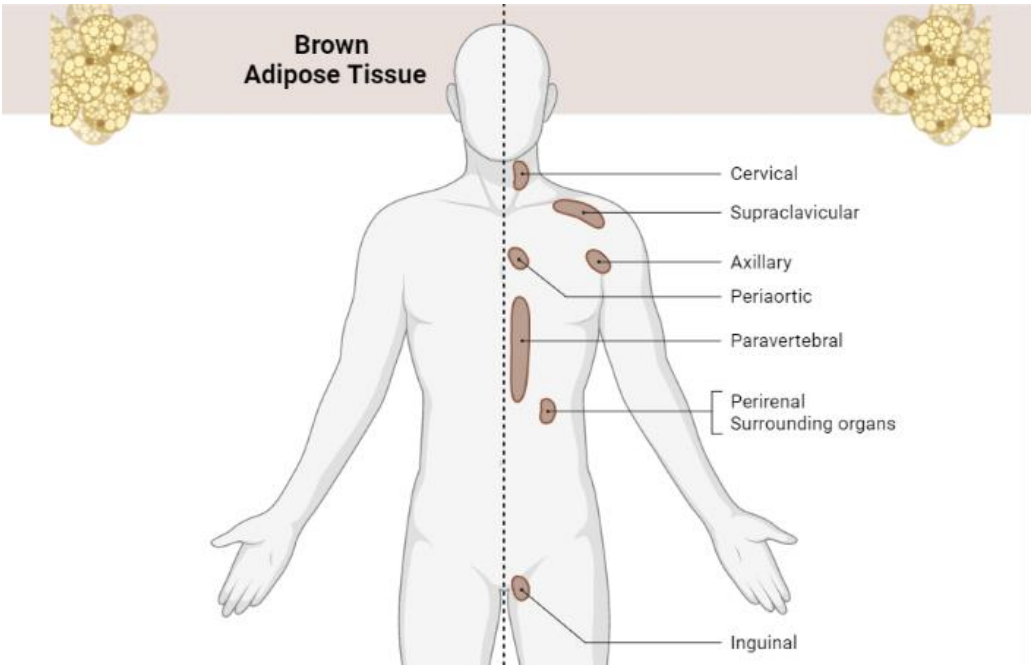
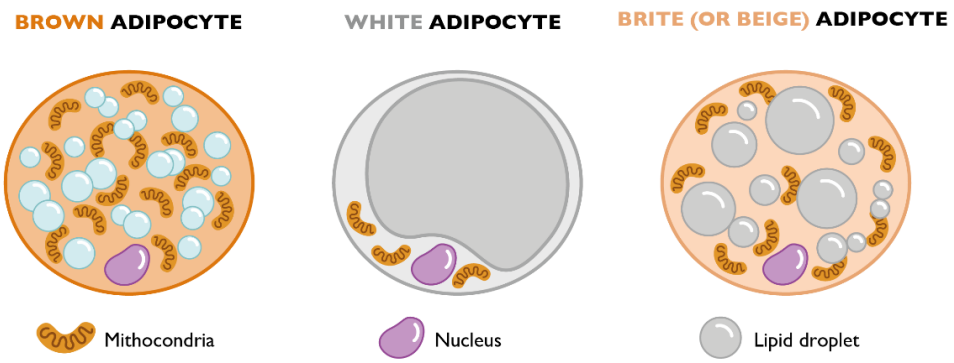


Figure 3. *WAT and BAT depots localization in human adults. Adapted from "Adipose Tissue Depots", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates*

BAT seems to be inversely associated with age[21,25–27], BMI[21,26], and visceral adiposity[22,25]. However, these assumptions have been disputed and might be related to bias in the assessment methods[29]. Therefore, more studies are needed to clarify the relationship between BAT and adiposity [30].

During the last years, another type of cell has been found in the WAT of rodents and humans[31]. These cells are called brown-in-white (brite) or "beige" cells. These are characterized by possessing a similar morphology than brown adipocytes: multiple lipid droplets and enriched UCP-1-expressing mitochondria[31–34]. The process called as "browning" refers to the ability of white adipocytes to acquire a brown-like phenotype, so becoming beige adipocytes. The development of these cells is enhanced by chronic cold exposure and prolonged β-adrenergic stimulation, and it is also regulated by diverse factors in an endocrine paracrine and autocrine way [35]. **Figure 4** summarizes the differences and similitudes between white, beige and brown adipocytes.

Furthermore, the presence of these cells seems to be associated with protection against obesity, T2D, and other metabolic diseases [36]. Interestingly, the molecular signature of supraclavicular BAT depots in humans is more similar to these beige cells rather than to the murine brown adipocytes [37,38].



	BROWN	WHITE	BRITE
UCP1 Expression	Positive	Negative	Positive
Mitochondrial Density	High	Low	Medium
LD Morphology	Multi-locular	Uni-locular	Medium
Primary Function	Thermogenesis Endocrine	Energy-storage Endocrine	Thermogenesis? Endocrine?

Figure 4. *Main characteristics between brown, white and beige adipocytes. Adapted from Jung et al* [39]

BROWN ADIPOSE TISSUE ACTIVATION

Since BAT main function is to produce heat in order to maintain the core body temperature, cold exposure is the best stablished method to activate BAT[14]. Indeed, several studies in humans have observed a cold-induced increase in glucose uptake by BAT[15,40–44]. It has been reported that cold exposure also enhances blood flow[45,46], oxidative metabolism[45–47], intracellular lipolysis[16,47,48], and fatty acid uptake[47,48] in human BAT. Although to a lesser extent, it has also been observed that other stimuli can activate the tissue (e.g., diet[49] exercise[50]).

Cold exposure leads to SNS activation, promoting the release of norepinephrine by sympathetic nerve fibers and, consequently, the activation of β-adrenergic receptors (β–AR) of brown adipocytes. β-AR activation stimulates cyclic adenosine monophosphate (cAMP)-dependent signalling pathway, resulting in the stimulation of lipid catabolism and the expression of thermogenic genes, including UCP-1[51]. Recent studies have reported that although BAT thermogenesis in mice is mostly driven by β3-AR, it appears that in humans, this response should be mediated by β2-AR[52,53]. The pathway mediated by cAMP is the most studied model of BAT activation after cold exposure, although other two models have also been proposed[54]. **Figure 5** shows a simplified schematic of the UCP-1-dependent mechanism of brown adipocyte activation. Several UCP-1-independent mechanisms of thermogenesis have recently been proposed as well[55,56].

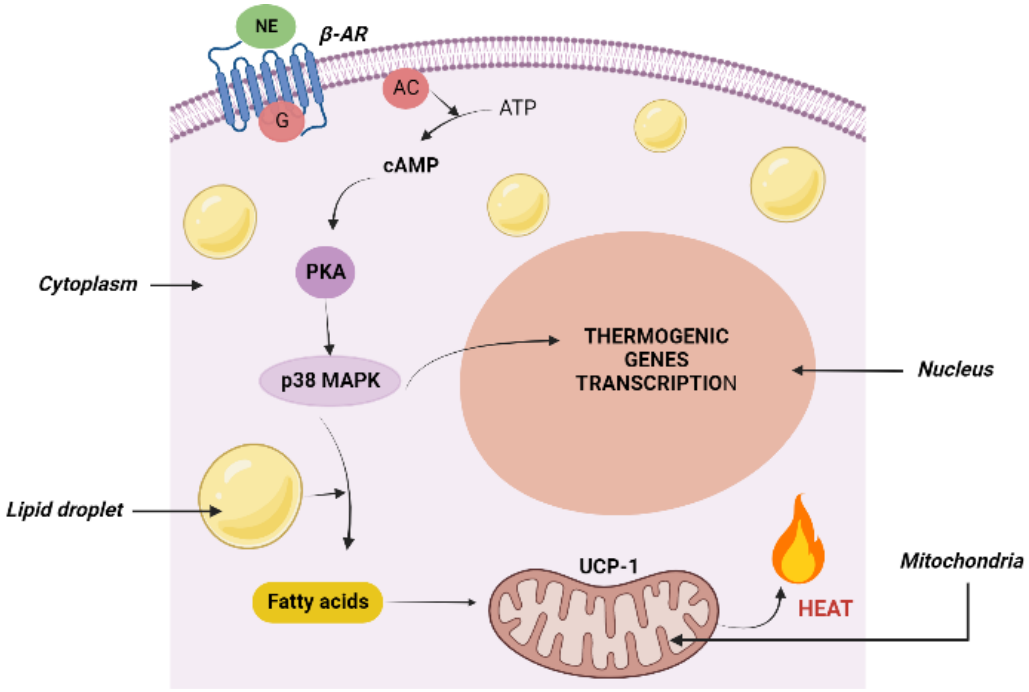


Figure 5. *UCP-1 mediated mechanism in BAT thermogenesis. Created with BioRender.com*

Fatty acids are the most relevant energy substrate to drive BAT thermogenesis, being brown adipocyte's triglycglycerols depots the main source[53]. Additionally, circulating non-esterified fatty acids (NEFA) and circulating triacylglycerols also contribute to thermogenesis after BAT activation when needed[53]. BAT glucose uptake after BAT thermogenesis activation is mostly used for lactate and triacylglycerol synthesis in brown adipocytes rather than thermogenesis substrate[57,58].

Considering its thermogenic capacity, BAT has long been considered as a strategy to prevent and combat obesity, playing a relevant role in whole-body energy expenditure[24,28,59,60]. Foster and Frydman (1978 and 1979) reported that BAT can account for up to 60% of total energy expenditure in rats when fully activated[61,62]. In humans, although our knowledge is limited, it is known that BAT involvement is much lower than in rodents, having a minimal impact on weight loss[45–47,63,64]. Another possible reason why BAT exerts a beneficial metabolic role could be the elevated metabolic rate and glucose uptake following BAT activation, which would represent a therapeutic target for T2D and insulin resistance states. However, it has been confirmed that, through shivering, muscle exerts a greater relevance in glucose oxidation and insulin sensitization after cold exposure[43,47].

Despite the relevant role of skeletal muscle in thermogenic capacity, several studies have suggested a beneficial effect of human BAT after cold exposure in thermogenesis and insulin sensitivity[42,43,65–67]. Moreover, different investigations have shown the relationship between BAT and human metabolism. Wibmet et al (2021) reported that BAT-positive subjects had lower visceral fat mass, lower liver fat content and lower T2D prevalence comparing with their

counterparts[68]. Similar results were observed by Becher et al (2021), where BAT-negative subjects were associated with lower T2D prevalence, dyslipidaemia and cardiovascular disease[69]. Nevertheless, these results should be interpreted with caution since a decrease in BAT glucose uptake may not be indicative of reduction of *in vivo* BAT thermogenesis.

Given that the scientific evidence to date is doubtful about the relevance of BAT in terms of total energy expenditure, it is plausible that this tissue exerts its beneficial role through additional mechanisms. Interestingly, in recent years it has been revealed that the therapeutic function of BAT observed both in mice and humans should be due to its secretory role, as it will be discussed in the next section.

THE SECRETORY ROLE OF BROWN ADIPOSE TISSUE

Since the discovery of leptin[70], several WAT-secreted molecules involved in metabolism, called adipokines, have been discovered[71]. However, the majority of the WAT-secreted adipokines are poorly expressed in BAT (e.g., leptin[14]), so little attention had been focused on the endocrine role of BAT. BAT secretes multiple molecules, known as batokines or brown adipokines, which exert autocrine, paracrine and endocrine actions[35] (**Figure 6**). Some examples of molecules with autocrine and paracrine roles are fibroblast growth factor 2 (FGF2) and insulin-like growth factor 1, which stimulate proliferation and differentiation of preadipocytes in BAT[72,73]. Nerve growth factor (NGF) is suggested to promote sympathetic innervation and hyperplasia of BAT, required for BAT thermogenic recruitment and thermogenic capacity enhancement[74–76]. Another example is the vascular endothelial growth factor A (VEGFA), which promotes BAT vascularization[38,77]. *Vegfa* overexpression in mice upregulates *Ucp-1*, increasing thermogenic response after chronic cold exposure[78]. Other BAT-secreted proteins with autocrine and paracrine actions are bone morphogenic proteins (BMPs), relevant for brown and beige adipocytes differentiation[79–81].

In addition to the aforementioned molecules, mostly implied in BAT activation thermogenic response and WAT browning, there are also some BAT-secreted proteins implied in immunometabolism. It is known that the dramatic increase in proinflammatory cytokines production in WAT is associated with obesity, with a wide number of infiltrating proinflammatory immune cells (mostly macrophages) in the tissue[82]. This inflammatory state is associated as well with insulin resistance, implied in multiple disorders, including T2D[82]. In contrast, and although immune cells in BAT have been still poorly explored, the number of BAT-secreted proinflammatory cytokines is lower, reflecting an anti-inflammatory phenotype in comparison with WAT[83,84]. Interestingly, the BAT-secreted anti-inflammatory cytokines have been associated with thermogenic adaptation[83,84], and alternatively-activated macrophages M2 have been

associated with BAT recruitment and activation and WAT browning [84–87]. Nevertheless, this research field needs to be further explored in humans.

Finally, BAT is also known to release factors with an endocrine role, targeting other tissues and promoting cardiometabolic benefits[35]. In recent years, the potential secretory role of BAT has been intensively researched[35,88,89], being triiodothyronine (T3) the first recognized BAT-released hormone[90], contributing to the systemic levels of T3[90,91]. Interestingly, a recent study has identified at least 471 proteins in the secretome of human BAT, of which 101 are not found in human subcutaneous abdominal WAT[92]. The relevance of BAT endocrine function has been evidenced by genetic BAT ablation[93–95] and BAT transplants in murine models[35,96–98], highlighting the relevance of BAT-released molecules with an endocrine role such as interleukin 6 (IL-6). These studies have consistently shown weight loss, improvement of glucose and insulin homeostasis, and cardioprotective effects, among other benefits [99]. The endocrine role of BAT is widely exposed in Chapter 1.1 of Results Section.

Despite the scientific progress in recent years on the BAT secretome, most of the available information comes from rodents, and how BAT contributes to the systemic levels of batokines and its relevance at the physiological level in humans remains to be clarified. This field of research may be especially relevant for the use of circulating biomarkers in BAT activity or for the development of new pharmacological drugs to treat obesity and comorbidities. Considering the potential therapeutic application of this research topic, one of the purposes of this Doctoral Thesis is to identify circulating human batokines following cold exposure and to observe the associations of these cold-induced changes with BAT volume and activity. This aim is addressed in Chapter 1.2.

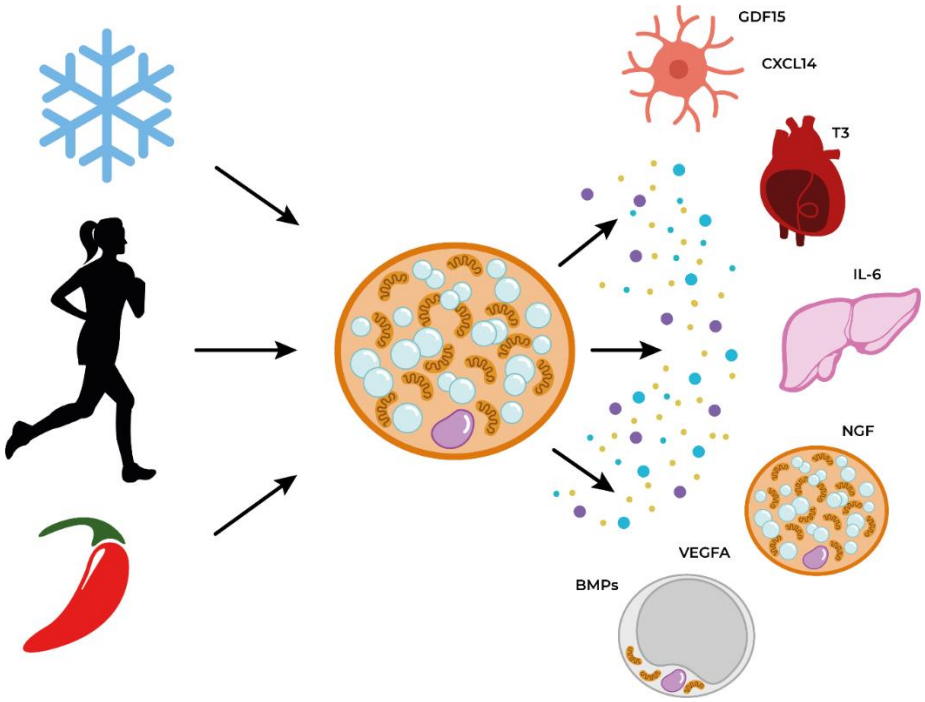


Figure 6. *The secretory role of BAT.*

THE EFFECT OF EXERCISE ON HUMAN BAT AND WAT BROWNING

Due the beneficial effect of BAT on metabolism, efforts are being put into trying to find alternative stimuli that can activate BAT, in the same way that cold exposure does. According to scientific evidence performed in murine models, exercise could be an additional activator of BAT.

Exercise is defined as a category of physical activity (i.e., movements produced by skeletal muscle resulting in energy expenditure[100]) with the requirement to be structured, planned, repetitive, and purposeful in relation to physical fitness[101]. It is widely known the enormous potential of exercise as therapy for several diseases, including cardiometabolic diseases, dislypidemia, depression and T2D, among others[102]. Nevertheless, the underlying mechanisms by which exercise promotes cardiometabolic benefits are not completely understood[103]. In recent years, studies in rodents have demonstrated that exercise may be a potential stimulus for BAT recruitment and activation as well as WAT browning. This effect is mediated by the promotion of sympathetic activity, the release of adrenergic factors and numerous endocrine signals, known as exerkines (e.g., irisin, fibroblast growth factor 21 (FGF21), etc.)[50,95,104,105]. The exerkines identified to date are reviewed in Chapter 2.1.

Despite the available literature, if exercise modulates human BAT is not completely clear[106–110]. Findings from observational studies are contradictory. Some authors reported no association of objectively measured physical activity[111] or fitness[112] with BAT volume or activity after a personalized cold exposure in young healthy adults. In contrast, other authors reported a positive association of subjectively measured physical activity with thermoneutral BAT activity in cancer patients[113] and a higher expression of browning markers in abdominal subcutaneous WAT[114]. Findings from case-controlled studies show that endurance trained athletes present lower BAT glucose uptake than their untrained counterparts[106,108,109], whereas no between-group differences in abdominal subcutaneous WAT browning markers expression were observed [108].

For years, interventional studies in humans studying the effect of exercise on BAT activity and volume have been controversial and inconclusive[107,115–117]. Some of the methodological problems were the small sample size, the lack of a control group to avoid cofounders such as seasonality, or the failure to provide data on BAT volume and activity before and after the exercise intervention. However, a 24-week randomized controlled trial with 97 participants has been recently published by Martinez-Tellez et al (2020)[118]. In this study, young sedentary adults were assigned to three different groups (control, moderate intensity exercise and vigorous intensity exercise) and followed a 24-week supervised exercise intervention combining endurance and resistance exercise. Interestingly, authors reported no changes in BAT volume or activity after the intervention, measured by ^{18}F-fluorodeoxyglucose uptake, concluding that the observed exercise-benefits were independent of BAT.

Further research is needed for a better understanding of the role of exercise and BAT activation. In addition, it would be fascinating to ascertain the involvement of different types of exercise, intensities and duration, given that most of the studies relating exercise to BAT have been carried out just in chronic exercise. Furthermore, regardless of the effect that exercise has on human BAT, it seems plausible that exercise stimulates the release of various endocrine factors into the bloodstream which are modulators of BAT activity and activators of the WAT browning process [105]. For this reason, the present Doctoral Thesis aims to study the endocrine mechanisms involved in the activation of BAT through different types, intensities and duration of exercise in human adults, as it will be show in Chapter 2.2 of the Results Section.

AIMS

Overall aim

The overall aim of this Doctoral Thesis is to study the endocrine role of human BAT, as well as the exercise-released factors capable to activate BAT and WAT browning in sedentary young adults.

Specific aims

- **Chapter 1.2**, **Study I** – To study the endocrine role of BAT in sedentary young adults.
  - Aim 1.1. To investigate the effect of a 2-hour individualized cold exposure on the plasma levels of 5 batokines in sedentary young adults.
  - Aim 1.2 To analyse whether cold-induced changes in circulating batokines are related to BAT function in sedentary young adults.

- **Chapter 2.2, Study II** – To examine the endocrine factors released by exercise and its relation with BAT function in sedentary young adults.
  - Aim 2.1. To quantify in sedentary young adults, the circulating levels of 16 exerkines after three different types of exercise: i) acute endurance exercise, ii) acute resistance exercise, iii) chronic exercise.
  - Aim 2.2 To analyse the association between the exercise-induced changes in circulating exerkines and BAT function in sedentary young adults.

METHODOLOGICAL OVERVIEW

METHODOLOGICAL OVERVIEW OF THE DOCTORAL THESIS

The present Doctoral Thesis is composed in two different chapters. Both chapters start with a broad theoretical framework, followed by Study I and Study II, respectively. All the studies have been performed under the framework of the Activating Brown Adipose Tissue Through Exercise (ACTIBATE study). **Study I** aims to analyze the endocrine role of BAT during an individualized cooling protocol, and **Study II** investigates the effect of different type of exercise on several circulating factors that could be modulators of BAT metabolism and WAT browning.

Table 2. *Methodological overview of the studies included in the Doctoral Thesis.*

Study	General Aim	Design	Participants	Main Study outcomes	Statistical analyses
I	To study the endocrine role of BAT in sedentary young adults.	Longitudinal	N=30 (♀ 60%)	Plasma levels of 5 batokines BAT variables (volume, SUVpeak, mean radiodensity)	Repeated measures analyses of variance Simple and multiple linear regressions
II	To examine the endocrine factors released by exercise and its relation with BAT function in sedentary young adults.	Longitudinal + Cross-sectional	Discovery study *(acute)* N=10 (♀ 60%) Confirmatory study *(acute)* N=38 (♀ 74%) Chronic study N=110 (♀ 68%)	Plasma levels of 16 exerkines, BAT variables (volume, SUVmean, SUVpeak, mean radiodensity)	Repeated measures analyses of variance Analyses of covariance Simple and multiple linear regressions

THE ACTIVATING BROWN ADIPOSE TISSUE THROUGH EXERCISE (ACTIBATE) STUDY: DESIGN AND METHODOLOGY

Study design

The ACTIBATE study [118,119] was randomized controlled trial designed to study the effect of an exercise program on BAT volume and activity (ClinicalTrials.gov ID: NCT02365129). This study was conducted following the last version of Declaration of Helsinki. The protocol and written informed consent were approved by the Ethics Committee on Human Research of the University of Granada (nº 924) and "Servicio Andaluz de Salud".

Participants of the study were randomly assigned in three different groups: i) control; ii) moderate-intensity exercise and iii) vigorous-intensity exercise. All baseline evaluations were performed at the Instituto Mixto Deporte y Salud (IMUDS) at the University of Granada and the Hospital Universitario Virgen de las Nieves, Granada, Spain, by the same researchers. The study had a duration of 6 months. Baseline examinations were assessed between October and November 2016 (≈60 participants) and 2016 (≈90 participants).

Participants recruitment and selection criteria

To recruit the participants, the study was divulged on local media, social networks, and posters at the Faculties of the University of Granada and information sessions were offered. Those people who were interested in being participant of the study contacted through social networks and visited the research centre to be widely informed about the study characteristics and procedures. A short online survey about eligibility criteria was filled by interested individuals (i.e.: age, height, present and past weight, physical activity, medication, current medical history, smoking, alcohol habits, and residence). On a second meeting, interested people obtained a detailed written information about the study and eligible participants understood and signed the informed consent. The inclusion and exclusion criteria are showed in **Table 3**.

Table 3. *Inclusion and exclusion criteria for ACTIBATE study.*

Inclusion criteria	❖ 18-25 years. ❖ 18.5-35 kg/m^2. ❖ <20 min of moderate-vigorous physical activity on <3 days/week ❖ Not participating in a weight loss programme. ❖ Stable body weight over the less 3 months (<3kg). ❖ Normal electrocardiogram. ❖ Capable to understand and willing to provide consent and accept the randomized group assignment.
Exclusion criteria	❖ History of cardiovascular disease. ❖ Diabetes or hypertension. ❖ Pregnancy or planning to get pregnant during the study period. ❖ Medication that impact their cardiovascular or thermoregulatory responses to cold exposure. ❖ Smoking. ❖ Frequent exposure to cold temperatures. ❖ Unwillingness to either complete the study requirements or to be randomized into control or training group. ❖ First-degree relative with history of cancer.

Baseline assessments of ACTIBATE study

A medical examination was performed in the first visit. After that, baseline assessments were performed in 8 different days regardless of the order. The different tests are shown in **Table 4**. These outcomes were measured again after the 24-week intervention program. The **Figure 7** shows the flow-chart of the participants finally included in the ACTIBATE study.

***Table 4**. Baseline assessments performed in ACTIBATE study.*

Day	Tests and outcomes
1	❖ Muscle strength test and online questionnaires about health and life quality.
2	❖ Heart rate variability during 15 min. ❖ Energy expenditure trough indirect calorimetry. ❖ Body composition.
3	❖ Fasting blood samples and muscle and adipose tissue biopsies in overnight fasting condition.
4	❖ Maximum graded exercise test under medical supervision. ❖ Blood pressure, respiratory gases interchange and electrocardiogram after the exercise test.
5	❖ Submaximal walking effort test in a fasting condition to determine maximal fat oxidation. ❖ Respiratory gas exchange during the test.
6	❖ Shivering threshold test (STT).
7	❖ Positron emission tomography-computerized tomography (PET-CT) scans to determine BAT mass and activity after cold exposure. ❖ Blood samples at thermoneutral and after cold exposure (1h and 2 h). ❖ Blood samples during an endurance exercise session and another strength exercise session.
8	❖ 48-72 hours later than the PET-CT, CIT and CI-NUTox assesed to measure resting metabolic rate (RMR).

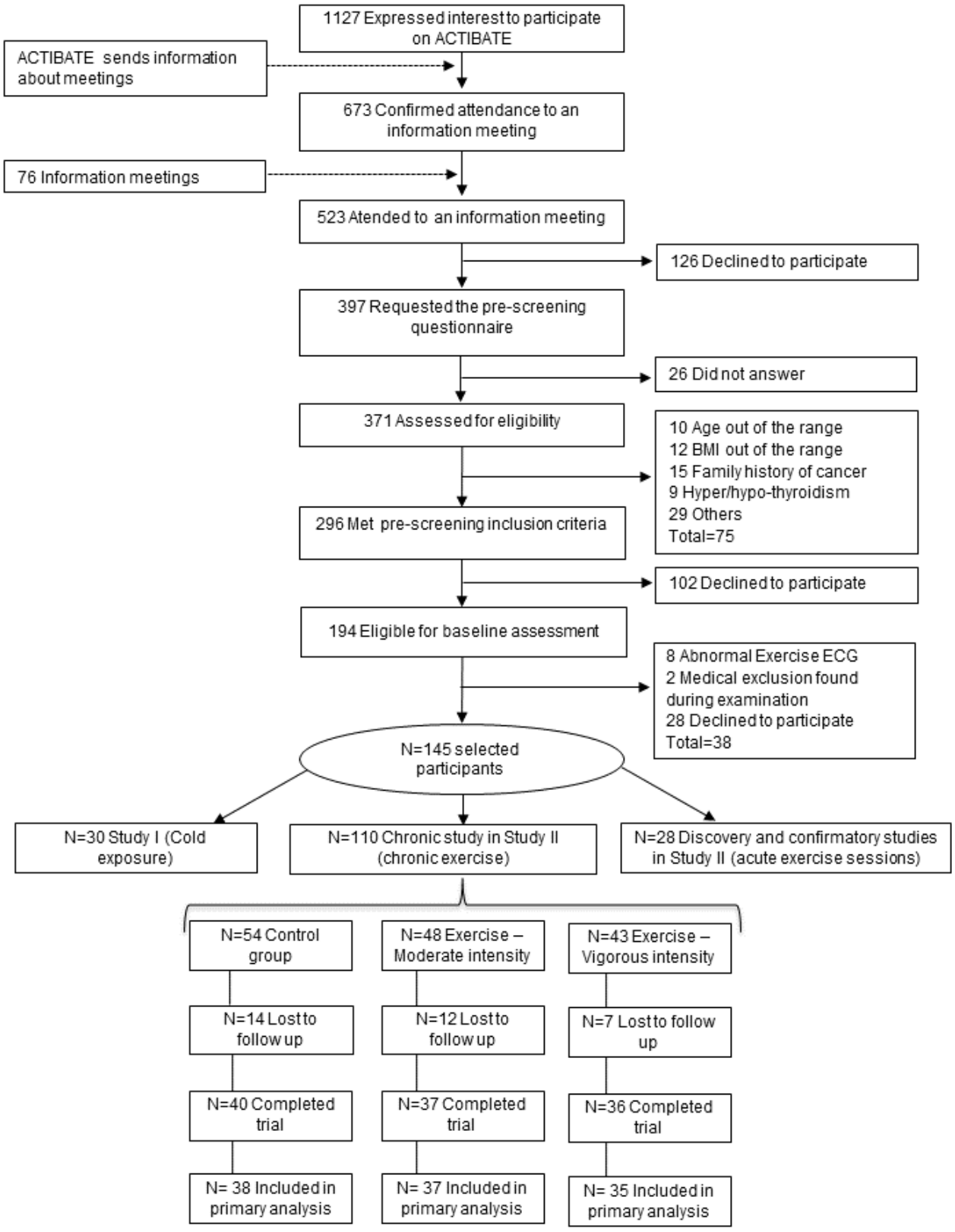


Figure 7*. Flow-chart of ACTIBATE study included in the Present Doctoral Thesis*

RESULTS AND DISCUSSION

CHAPTER 1. Endocrine role of brown adipose tissue

1.1. Brown adipose tissue as an endocrine organ: batokines and metabolic relevance

Beyond the thermogenic capacity of BAT, its potential secretory role has been intensively investigated in recent years. BAT secretes multiple molecules, known as brown adipokines or batokines[89], and some of them can target distant cells and organs[88]. The relevance of its endocrine function has been mainly evidenced by BAT transplants in preclinical models. These studies have consistently shown several benefits such as weight loss, improvement of glucose and insulin homeostasis, and cardioprotective effects, among others[99]. Indeed, some studies have reported that a small amount of embryonic BAT had reversed type-I diabetes in adult rodents[120,121]. In addition to the evidence observed with BAT transplantation, more pronounced systemic effects have also been observed in those mouse models with genetic ablation of BAT compared with those with just thermogenic BAT impairment[93]. The identification of genes encoding proteins secreted by BAT in response to thermogenic stimuli has also put the spotlight on the endocrine function of the tissue[88]. This section reviews the state of the art of the possible batokines identified to date.

Chemokine C-X-C motif chemokine ligand 14 (CXCL14)

Recent studies have reported the relevance of immune cells in BAT and beige adipose depots, highlighting the involvement of M2 macrophages recruitment in the thermogenic response[122,123]. Nevertheless, the mechanisms of M2 recruitment in these tissues remain controversial. Cereijo et al (2018)[124] observed that CXCL14 plasma levels were increased in mice after thermogenic stimuli. In this study, the authors observed that this increase was positively associated with an induction of macrophage recruitment, mostly M2 phenotype, and browning of subcutaneous WAT. Interestingly, when CXCL14 expression was suppressed, BAT activity was impaired and recruitment of M2 macrophages in BAT was attenuated[124]. Thus, this molecule represents an example of the crosstalk between BAT and WAT, inducing an anti-inflammatory state in the tissue and the browning process. Recently, Garcia-Beltran et al (2022) have identified high *CXCL14* expression levels in neonatal BAT compared to placenta, liver and WAT, which may indicate that CXCL14 is also expressed in human BAT[125].

In relation with the metabolic effects of CXCL14, results are controversial[126,127]. Nevertheless, Cereijo et al (2018) reported a consistent positive association between CXCL14 plasma levels and improved glucose homeostasis in different animal models[124]. In this study, the authors observed a recovery of CXCL14 serum levels after bariatric surgery in men, as well as a reduction of circulating CXCL14 levels in mice fed a high-fat diet (HFD), suggesting to have a role in glucose homeostasis[124]. In addition, Cereijo et al (2021) reported that serum levels of

CXCL14 were decreased in patients with obesity, and specifically, in patients with type-2 diabetes[128]. Moreover, they observed a negative correlation between *CXCL14* expression in WAT and genes encoding pro-inflammatory molecules, as well as a positive correlation between *CXCL14* expression and *GLUT4* expression. These findings highlight the possible role of CXCL14 in the control of glucose metabolism and the inflammatory state of WAT. If CXCL14 would confirmed to be a human batokine and its endocrine role in adipose tissue's immune cells and glucose metabolism, it would constitute a possible strategy for the prevention and treatment of metabolic diseases.

Growth differentiation factor 15 (GDF15)

It has been recently discovered that GDF15, a member of the transforming growth factor β superfamily (TGFβ), is released by brown adipocytes after cold exposure or noradrenergic stimulation *in vitro*[129,130]. Interestingly, Campderrós et al (2019) observed that the FGF21 pathway is needed for noradrenergic regulation of GDF15[130].

GDF15 has shown to exert mostly autocrine actions on macrophages, inducing M2 phenotype and, consequently, an anti-inflammatory effect, downregulating local inflammatory pathways[130]. However, if the cross-talk between immune cells and BAT-released GDF15 is just limited to local effects in adipose depots or have consequences on the systemic immune status remains ascertained. Interestingly, this molecule has also been suggested to have an anorexigenic effect and a role in controlling the energy balance in mice[131,132].

Fibroblast growth factor 21 (FGF21)

FGF21 is known to be a hormone with beneficial effects on the control of glycaemia, lipid metabolism and insulin sensitivity[133]. It is implicated in the thermogenic response of BAT exerting autocrine and paracrine actions[134]. Although the liver is the main source of systemic FGF21 in most conditions[135], brown adipocytes express and release this hormone after thermogenic stimuli through noradrenergic and cAMP-mediated mechanisms[136,137]. Intriguingly, Hondares et al (2011) identified BAT as the source of systemic FGF21 production after thermogenic activation in rodents through noradrenergic control of *Fgf21* gene transcription[137]. In the study, they also observed a positive association between the cold-induced increase in plasma levels of FGF21 and the protein release by BAT, assessed with arteriovenous differences in plasma FGF21 levels across interscapular BAT[137]. In another study performed in 2014, Hondares et al reported that *FGF21* and *UCP1* were expressed in visceral and interscapular fat in human neonates, showing a positive correlation between them[138]. Studies in humans have also confirmed an increase in FGF21 plasma levels after cold stimulation. Hanssen et al (2015) found that serum FGF21 levels at baseline levels were positively correlated with BAT activity during acute cold exposure in male subjects, measured by ^{18}F-FDG PET-CT scan[139]. Soundarrajan et al (2020) also

observed a positive correlation between serum FGF21 and BAT SUV_{max} in young adult men after an individualized cooling protocol prior to ^{18}F-FDG PET-CT imaging[140].

The metabolic protective effects exerted by FGF21 is illustrated by the tissues expressing its receptor, which are mainly the heart, WAT, brain and pancreas[138]. Ruan et al (2018) observed that FGF21 cardioprotective effects seems to be mediated by BAT, as these effects were attenuated in brown adipocyte-specific FGF21 knockout mice[94]. Additionally, in the same study, the authors reported that recombinant FGF21 administration improved cardiac remodelling in intercapsular BAT-depleted hypertensive mice[94]. Furthermore, in another study, it was reported that *UCP-1* null mice showed myocardial injury and adverse cardiac remodelling[141]. Taken together, these findings could provide evidence for a crosstalk between BAT activity and cardioprotective actions through BAT-released FGF21 and the endocrine role of this protein on the heart. Although more studies are needed to understand the human complexity in comparison with murine models, FGF21 may be an attractive tool for BAT activation and, consequently, a potential strategy for prevention and treatment of cardiac disease, among others.

Interleukin 6 (IL-6)

Cold exposure or noradrenergic stimulation leads an increase of *IL-6* expression and IL-6 release by brown adipocytes[142]. Although IL-6 is a well-known proinflammatory cytokine, it has been shown that IL-6 is capable to target cells and sensitize them to the action of IL-4, promoting a M2 macrophage activation[143,144] and inducing an anti-inflammatory status. This effect is known to play a role in improving insulin sensitivity in WAT[144,145] and is suggested to enhance hepatic gluconeogenesis[146].

It is known that IL-6 targets not only the liver, but also the brain and pancreas[147]. Interestingly, Stanford et al (2013) showed that although a transplant of a small amount of adult BAT into mice with diet-induced obesity reversed the insulin resistance and obesity, the effect was attenuated when the BAT donor was IL-6 null mice[145]. This observation highlights the effect of this molecule in BAT cardiometabolic benefits. In addition, they observed that BAT transplant in mice increased FGF21 serum levels, while IL-6 null BAT donors blunted this increase, which suggest that IL-6-effects of BAT on metabolism may be mediated by FGF21 secretion and action. Assuming that IL-6 is indeed a human batokine, it would be of considerable therapeutic value due to the wide variety of tissue targets it would offer, improving the cardiometabolic status of patients.

Bone morphogenic protein 8

Bone morphogenic protein 8 (BMP8b), a member of the TGFβ family, was first identified as a batokine in 2012[148]. Garcia-Beltran et al (2022) have recently found that human neonatal

BMP8B mRNA levels were higher in BAT that in WAT, liver and cord blood[149]. Moreover, in this study, BMP8b circulating levels were high at birth and declined progressively over the first year of life, which might reflect the evolution of BAT activity[149]. Additionally, Urisarri et al (2021) have observed that BMP8b circulating levels in neonates were associated with the BAT thermogenic response, measured with infrared thermography after a single short-term cold stimulus[150]. Overall, these data suggest that BAT may be responsible for BMP8b release to circulation in human neonates.

In addition to having paracrine and autocrine roles, BMP8b also exerts endocrine actions[151,152]. Whittle et al (2012) reported that *Bmpb8b* is expressed in the hypothalamus of mice, and *BMP8b-/-* rodents showed altered neuropeptide levels, showing an anorexigenic state[148]. Additionally, the treatment with BMP8b activates BAT via AMPK in key hypothalamic nuclei, confirming the effect of this molecule on brain, at least, in rodent models[148]. New approaches are necessary to reveal whether BMP8b is a batokine, as well as to investigate its metabolic effects in human adults.

MicroRNAs (miRNAs)

Several studies have reported a high expression of some miRNAs (e.g.: miR-193b and miR-365, among others) in BAT but not in WAT or muscle, showing a relevant role in the brown adipocyte differentiation[153,154]. Interestingly, Trajkovski et al (2012) observed that miR-133, a negative regulator of PRDM16 expression, is downregulated in BAT after cold exposure, resulting in a brown adipocyte phenotype[155].

Additionally to the role of miRNAs in brown adipocyte differentiation, it has recently been reported that BAT releases endosomes containing specific miRNAs capable to exert regulatory functions into the circulation[97,156]. The microvesicles secreted by cells, known as exosomes, contain miRNAs, small RNAs (20-22 nucleotides in length) capable to modulate gene regulation through silencing specific mRNAs[157,158]. Interestingly, when BAT is thermogenic activated, circulating exosomes containing miR-99b are increased[97] whereas those containing miR-92a are reduced[156]. Chen et al (2016) reported an inverse association between serum levels of miR-92a and human BAT activity measured by ^{18}F-FDG PET-CT in 41 healthy individuals[156]. Thomou et al (2017) reported that BAT transplantation restored the circulating levels of miRNAs in a deficient miRNA mouse model, being also BAT the only adipose tissue capable to improve glucose tolerance in these rodents[97]. Moreover, in this study, the authors identified miR-99b as the one whose circulating levels significantly increased after BAT transplantation. Finally, they observed that miR-99b specifically modulates the hepatic production of FGF21, which may represent a BAT-liver crosstalk to activate BAT thermogenesis and maintain tissue activation[97]. The BAT-released microRNAs in humans should be further investigated. The identification of

these molecules would be a potential strategy both for their use as biomarkers of BAT activity and for the development of pharmacological strategies in the field of cardiometabolic diseases.

Lipokines

Lipokines are lipid species that have recently been reported to exert endocrine functions, for example after cold stimulation[89]. Some of them have been identified as being released by BAT; and are therefore considered as potential batokines. Examples are 12,13-dihydroxy-9Z-octadecenoic acid (12,13-diHOME) and 12-hydroxyeicosapentaenoic acid (12-HEPE).

Although underlying mechanisms are yet unknown, Lynes et al (2017) reported that circulating 12,13-diHOME was increased after cold exposure in humans and mice[159]. They also observed in this study that the injection of 12,13-diHOME acutely activated BAT fuel uptake, decreasing serum TGs and was also negatively correlated with BMI and insulin sensitivity in mice[159]. Interestingly, it has been observed that 12,13-diHOME is released by BAT in response to exercise in humans and mice[96,98]. The surgical removal of BAT in mice impaired the increase of 12,13-diHOME after exercise, suggesting that BAT is the main source of this lipokine[96]. The 12,13-diHOME treatment in mice was capable of inducing fatty acid uptake and oxidation in skeletal muscle and thermogenesis in BAT[96]. Therefore, this lipokine seems to orchestrate an inter-organ communication between BAT and muscle. In relation with other systemic effects of 12,13-diHOME, Kelsey et al (2021) have recently identified a direct role of BAT on cardiac function mediated via 12,13-diHOME in a murine model of BAT transplantation[98]. In this study, BAT transplantation improved cardiac function through targeting directly cardiomyocytes, improving cardiac hemodynamics and reducing the negative effects of a high-fat diet on cardiac function and remodelling[98]. The same authors also observed a negative correlation between 12,13-diHOME plasma levels in human patients with heart disease[98].

In addition to 12,13-diHOME, Leiria et al (2019) have also demonstrated that 12-HEPE is produced by brown adipocytes in mice[160]. In this study, they combined both serum and tissue lipidomics from different murine models both *in vitro* and *ex vivo* and reported that BAT is the source of 12-HEPE after cold exposure. Moreover, they also suggested an endocrine role of 12-HEPE as a batokine in this study. They reported that this lipokine increased the expression of lipogenesis genes and *Glut-1* in murine BAT, leading Glut-4 translocation to the plasma membrane and, consequently, the glucose uptake into brown adipocytes. Furthermore, they observed an increase in glucose uptake after 12-HEPE administration *in vivo* in human brown adipocytes and in murine C2C12 myotubes, providing evidence that 12-HEPE could exert an endocrine role in human BAT and skeletal muscle on the control of glucose metabolism.

Neuregulin-4 (Nrg4)

Neuregluin-4 (Nrg4) is a member of the epidermal growth factor family of extracellular ligands, proposed as a BAT-released factor capable to protect against diet-induced insulin resistance and hepatic steatosis in murine models[161], also associated with a healthy metabolic profile in women with gestational diabetes mellitus[162]. Nrg4 has also been proposed to induce terminal nerve branching[163]. This molecule was identified in transcriptomic microarray assays and it has been predicted that it may be responsible for the secretion of proteins induced during brown adipocyte differentiation[163] However, it has not been detected in some proteomic studies in murine adipocytes[164,165]. This may be due to the low concentration ranges of this molecule or the potential limitations of the proteomics technology as well. Further studies in human should be considered to determine if Nrg4 can be considered as a human batokine as well as its endocrine role after BAT activation.

Ependymin-related protein 1 (EPDR1)

Ependymin-related protein 1 (EPDR1) has recently discovered to be present in human brown adipocytes secretome through a proteomics-based study, being proposed as a novel batokine[92]. In the same study, authors detected EPDR1 in human plasma samples, suggesting an endocrine role and a crosstalk between tissues[92]. Moreover, *EPDR1*-silencing in human brown adipocytes decreased norepinephrine-induced mitochondrial proton-leak respiration, also altering the expression of mitochondrial proteins[92]. In order to discover new metabolic effects of EPDR1, Rodrigo et al (2022) studied the role of EPDR1 in β-cell metabolism in human pancreatic islets from healthy and T2D participants[166]. They found that EPDR1 expression might enhance glucose-stimulated insulin secretion in obesity. Thus, up-regulation of EPDR1 expression in obese individuals may result in a decreased risk of glucose intolerance. It is important to develop further studies in humans that can confirm the role of EPDR1 as a batokine and its endocrine role in glucose metabolism, improving the clinical status of patients with cardiometabolic diseases.

Myostatin

Myostatin has been classically considered as a myokine, a molecule secreted by muscle and involved in the negative regulation of muscle mass, downregulating myogenesis genes[167]. Surprisingly, Kong et al (2018) reported that BAT contributes significantly to serum myostatin levels, at least in mice[95]. In this study they observed a rise in myostatin mRNA and protein in BAT but not in skeletal muscle, accompanied by an increase of myostatin circulating levels in warming conditions. If circulating levels of myostatin are upregulated after BAT activation in humans is unknown. In addition to the possible crosstalk between BAT and skeletal muscle through myostatin released, this molecule has also been attributed a negative regulatory role in myocardial mass[168]. Indeed, serum myostatin levels have been observed to be elevated in patients

with heart failure [169]. If this relation was confirmed, myostatin could also be used as a predictor of myocardial scar burden.

As reviewed in this section, BAT is a secretory organ capable of releasing into the bloodstream numerous molecules, called batokines, that can target remote tissues (**Figure 8**). It has been seen that these batokines exert beneficial actions on metabolism, acting at a cardioprotective level, improving insulin and glucose metabolism or encouraging an anti-inflammatory environment, among others. Nevertheless, most of the studies available so far have been carried out in murine and *in vitro* models and whether human BAT significantly contribute to increase circulating levels of batokines remains ascertain. Therefore, additional human studies are required since translating findings of BAT physiology from rodents to humans is quite challenging. It would be highly relevant not only the discovery of human batokines, but also their targets and actions on human metabolism.

The identification of human circulating batokines and the characterisation of their effects are expect to improve our knowledge of potential tools and targets to prevent and treat obesity and associated metabolic diseases. Scientific progress in this field of research would allow, on the one hand, the detection of circulating biomarkers of BAT activity, as well as allowing an opportunity for the development of pharmacological strategies to mimic the actions of batokines in metabolism. In fact, there are groups that are currently working on the development of drugs that increase FGF21 levels to treat liver diseases, or type II diabetes[170,171]. One of the purposes of the present Doctoral Thesis is to contribute translational research in the field of the batokines, studying 5 of them in plasma samples after cold exposure in adult humans. This Doctoral Thesis also aims to study the association of cold-induced changes with BAT volume and activity, trying to understand and elucidate whether human BAT is the possible source of the circulating batokines release. These objectives are addressed in Study I, in Chapter 1.2.

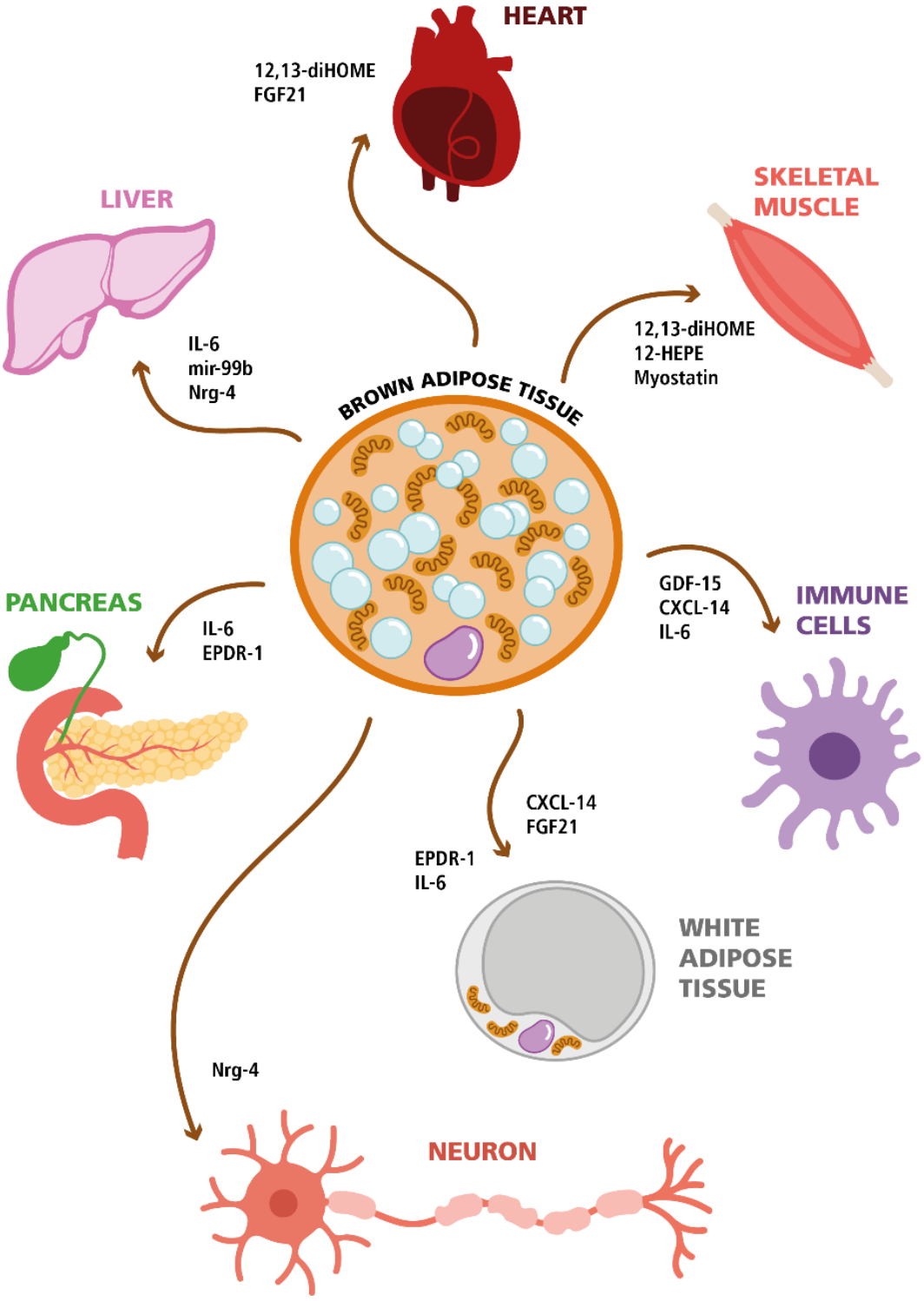


Figure 8. *Batokines released by BAT with endocrine role.*

1.2. Cold exposure modulates circulating batokines in humans, but only FGF21 is associated with brown adipose tissue volume

BACKGROUND

BAT secretes multiple molecules, known as brown adipokines or batokines [13], which exert autocrine, paracrine, and endocrine functions [14]. The relevance of its endocrine function has been manifested by BAT transplantation in preclinical models. These BAT transplants have consistently shown weight loss, improvement of glucose and insulin homeostasis, and cardioprotective effects, among other benefits [15–18]. These beneficial effects can hardly be attributed to the thermogenic activity of BAT explants, and are therefore hypothesized to be mediated by the BAT secretome. Thus, the identification of batokines in humans could represent a potential tool for the development of strategies to treat metabolic diseases such as obesity, diabetes, and cardiovascular diseases. Multiple preclinical studies have identified as batokines the chemokine ligand 14 (CXCL14) [19], growth differentiation factor 15 (GDF15) [20], fibroblast growth factor 21 (FGF21) [21], interleukin 6 (IL-6) [22], and bone morphogenic protein 8b (BMP8b) [23] . However, translating findings from rodent to humans is particularly challenging when it comes to BAT physiology [24]. Therefore, whether the secretion of these batokines by human BAT significantly contributes to the systemic pool needs to be elucidated. The present study is grounded on the hypothesis that, upon cold exposure -the best stimuli to activate BAT in humans- BAT should release a certain amount of these batokines to the circulation that may be detectable in plasma. If this assumption is true, the plasma levels of cold-induced batokines should be associated with BAT volume, ^{18}F-Fluorodeoxyglucose (^{18}F-FDG) uptake and/or radiodensity.

Therefore, the present study aims to determine, the effect of a 2-hour personalized cold exposure on the plasma levels of CXCL14, GDF15, FGF21, IL-6, and BMP8b, and their association with BAT volume, ^{18}F-FDG uptake and/or radiodensity in young humans.

Material and methods

Study design

This study was conducted under the framework of the ACTIBATE randomized controlled trial [25,26], which was designed to study the effect of an exercise program on BAT volume and activity (ClinicalTrials.gov ID: NCT02365129) [26]. Inclusion and exclusion criteria included being 18-25 years old, reporting no more than 20 minutes of moderate-vigorous physical activity on a maximum of 3 days per week, absence of cardiometabolic disease, being non-smoker, not being taking any medication affecting energy metabolism, and having stable body weight during the previous three months. This study was conducted following the last version of the Declaration of

Helsinki, and the protocol and written informed consent were approved by the Ethics Committee on Human Research of the University of Granada (nº 924) and "Servicio Andaluz de Salud".

The study design is summarized in **Figure 9**. A total of 30 participants (18 women and 12 men) were included in this study. 48-72 hours before collecting blood samples and assessing BAT by a static ^{18}F-FDG Positron Emission Tomography-Computeraized Tomography (PEC-CT), the participants' shivering threshold was determined. Then, a 2-hour personalized cooling protocol was used to stimulate BAT activity immediately before the PET-CT scan. Blood samples were collected before, and 1h and 2h after starting the cold exposure preceding the PET-CT to determine the plasma concentration of batokines.

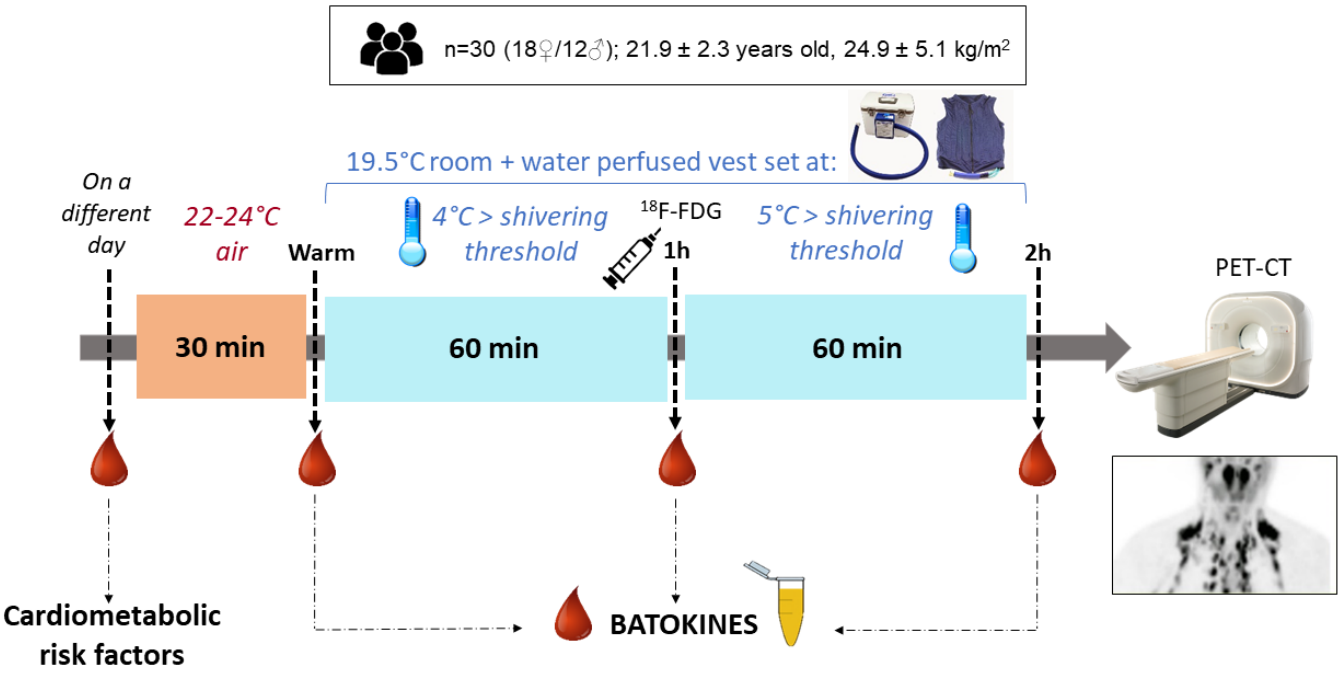


Figure 9. *Study design. Particpants were subjected to a 2-h individualized cooling protocol (using a water perfused vest adjusted to the individual's shivering threshold) while resting on a mild cold room. Blood samples were taken before and 1 and 2 hours after starting the cooling protocol. A bolus of ^{18}F-Fluorodeoxyglucose (^{18}F-FDG) was injected after 1 hour of cold exposure and 1 hour later, the Positron Emission Tomography-Computeraized Tomography (PET-CT) scan was perfomed.*

Shivering threshold test

The participants' shivering threshold was assessed to then personalize the cold exposure used to activate BAT before the PET-CT scan. To do so, participants first entered a warm room (22.1 ± 1.6ºC) for 30 min. After that, participants were transferred and remained seated into a room at 19.8 ± 0.5ºC, wearing a water-perfused cooling vest connected to a temperature-controlled chiller unit (Polar Products Inc., Ohio, USA). The vest covered the clavicular, chest, abdominal and back regions. The water temperature was initially set at 16.6ºC and was progressively reduced every

10-15 min until shivering onset (determined visually and/or self-reported) or a water temperature of 3.8ºC was reached. If participants did not shiver, they continued in the cold room, with the water temperature set at 3.8 ºC, additionally for 45 min. The individual shivering threshold was established as the temperature at which participants started to shiver [27].

Personalized cooling protocol

Two or three days after determining the shivering threshold, at the same time of the day, the personalized cooling protocol started with participants staying in a warm room (22.2 ± 0.5ºC) for 30 minutes. A peripheral catheter was inserted into the antecubital vein and blood was collected before starting the personalized cooling protocol. Immediately after, they were moved into a cold room (20.2 ± 0.3ºC) where they stayed seated wearing the cooling vest with the water temperature set 4ºC above the individual's shivering threshold. For those participants that did not shiver in the shivering threshold test, the water temperature was set at 3.8ºC [28]. After 1 hour of cold exposure, another blood sample was extracted, a bolus of ^{18}F-FDG (185 MBq; ~ 2.8 MBq/kg) was injected, and the water temperature was increased by 1ºC. If the subjects started to shiver at any time, the water temperature was immediately increased by 1ºC, and the subjects were covered with a bathrobe until shivering disappeared. After 2 hours of cold exposure a last blood sample was obtained and participants were transferred to another room where the PET/CT scan (Siemens Biograph 16 PET-CT system; Siemens, Berlin, Germany) was performed. Blood samples were collected in an EDTA-containing tube and were immediately centrifuged (10 minutes, 3000 rpm, 4ºC). Plasma aliquots were then stored at -80ºC until analyses.

PET-CT analysis

The PET image was obtained in two bed positions, from atlas vertebrae to mid-chest, while the CT was obtained by applying 120 kV [27]. PET-CT scans were analysed using the FIJI software as previously described [29], stablishing the regions of interest (ROI) from cervical vertebra 1 to thoracic vertebra 4 on both sides of the body (laterocervical, supraclavicular, mediastinum, and paravertebral). These ROIs were semi-automatically outlined and were computed as single ROI, as extensively described elsewhere [30,31]. The standardized uptake value (SUV) was calculated as ^{18}F-FDG uptake (kBq/mL))/(injected dose (kBq)/patient weight (g). Then, a SUV threshold of 1.2/(lean body mass/body mass) and a radiodensity range of -190/-10 were used to delineate BAT, following the BARCIST 1.0 recommendations [32]. Finally, BAT volume, SUVpeak and mean radiodensity were calculated by the software [29].

Plasma levels of batokines

The plasma levels of CXCL14, GDF15, FGF21 and BMP8b were determined using enzyme-linked immune-absorbent assay (ELISA) kits, according to the manufacturers' instructions.

CXCL14 was determined by ELH-CXCL14-1 (RayBiotech, USA, CV=9.2%), GDF15 by RD191135200R (Biovendor, Czech Republic, CV=6.8%), FGF21 by RD191108200R (Biovendor, Brno, Czech Republic, CV=7.11%), and BMP8b by MBS944757 (MyBioSource, San Diego, CA, United States, CV=8%).). IL-6 plasma levels were determined using XMAP technology by HSTCMAG-28SK (Luminex Corporation, Austin, TX, CV=11.5%).

Body composition

On a different day, body weight and height were measured using a SECA scale and stadiometer (model 799, Electronic Column Scale, Hamburg, Germany) while participants wore light clothing and were barefoot. Lean mass, fat mass, and visceral adipose tissue (VAT) mass were assessed by dual-energy x-ray absorptiometry (Discovery Wi, Hologic, Marlborough, MA). Waist circumference (WC) was measured twice with an elastic-plastic tape, and the mean value was obtained.

Cardiometabolic risk factors

Using blood samples collected on a different day, early in the morning after an overnight fasting, serum glucose, total cholesterol, high-density lipoprotein-cholesterol (HDL-C), and triacylglycerols were quantified using an AU5832 automated analyser (Beckman Coulter Inc., Brea CA, USA). Low-density lipoprotein-cholesterol (LDL-C) was estimated as [total cholesterol – HDL-C – (triacylglycerols /5)] [33]. Serum insulin was determined by the Access Ultrasensitive Insulin Chemiluminescent Immunoassay Kit (Beckman Coulter Inc., Brea, CA, USA) and the homeostasis model assessment of insulin resistance index (HOMA-IR) was calculated as [insulin(μU/ml) x glucose (mmol/L)/22.5]. The fatty liver index (FLI), a simple but accurate predictor of hepatic steatosis in the general population, was calculated using BMI, WC, triacylglycerols and gamma-glutamyl transpeptidase (GGT) levels, using the following equation [34]:

$$FLI = (e^{0.953*\log(\text{triacylglycerols}) + 0.139*BMI + 0.718*\log e(GGT) + 0.053*\text{waist circumference} - 15.745}) / (1 + e^{0.953*\log e(\text{triacylglycerols}) + 0.139*BMI + 0.718*\log e(GGT) + 0.053*\text{waist circumference} - 15.745}) * 100.$$

Systolic and diastolic blood pressure were measured twice on three different days using an automatic sphygmomanometer Omron M2 (Omron Healthcare, Kyoto, Japan), and the average of these values was used for the analyses.

Statistical analyses

Descriptive data are presented as mean ± standard deviation unless otherwise stated. The effect of the personalized cooling protocol on the batokines' concentration was analysed by repeated measures analyses of variance (ANOVA). The samples in which the batokines concentration was

below the range of detection (1 individual for CXCL14, 4 for GDF15, 6 for IL-6, and 3 for BMP8b) were given a value equal to half the magnitude of the lower end of the detection range an included in the analyses (although all the results remained unaltered when excluding these data). FGF21 and IL-6 did not follow a normal distribution. However, we observed similar results when performing the Friedman non-parametric test (data not shown). There were no *Sex x Time* interaction effects, therefore the analyses were conducted combining men and women. However, exploratory analyses were conducted separately in men and women, and similar trends were observed (data not shown).

To analyse the association between the cold-induced changes in batokines concentration and BAT-related variables, we calculated the change (Δ) in circulating levels of the batokines by subtracting the warm period value to the 1h and 2h cold exposure value. Then, we analysed, by simple linear regression (Model I), the association between both the Δ and the warm period batokines' concentrations, and BAT volume, SUVpeak, and mean radiodensity. These associations were also tested in multiple linear regression adjusting for the PET-CT scan date (Model II) as a proxy of the outdoor ambient temperature, sex (Model III), and BMI (Model IV). Similarly, we analysed the association between the plasma levels of the batokines and body composition and cardiometabolic risk factors.

Results

Participants were 21.92±2.1 years old, had a mean body mass index (BMI) of 24.9±5.1 kg/m^2 and a mean fat mass percentage of 24.9±8.3%. Additional characteristics are showed in **Table 5**.

***Table 5**. Characteristics of the study participants.*

	All (n=30)		Men (n=12)		Women (n=18)	
Demographics						
Age (years)	21.92	(2.3)	22.2	(2.5)	21.8	(2.3)
Body composition						
Weight (kg)	72.9	(18.2)	84.2	(21.1)	65.4	(11.3)
Height (cm)	171.1	(8.1)	177.2	(5.4)	166.2	(6.5)
Body mass index (kg/m^2)	24.9	(5.1)	26.7	(6.4)	23.7	(3.8)
Waist circumference (cm)	82.3	(14.8)	88.2	(18.9)	78.3	(9.9)
Lean mass (kg)	43.5	(10.4)	53.6	(8.3)	36.8	(4.4)
Fat mass (kg)	25.4	(98.8)	26.1	(13.1)	24.9	(7.5)
Fat mass (%)	34.9	(8.3)	29.9	(9.1)	38.4	(5.9)
VAT mass (g)	364	(215)	442	(244)	313	(182)
Cardiometabolic risk factors						
Glucose (mg/dl)	89.6	(8.4)	91.8	(10.7)	88.2	(6.4)
Insulin (µU/ml)	10.1	(9.3)	13.8	(13.1)	7.7	(4.4)
HOMA-IR	2.4	(2.6)	3.4	(3.7)	1.7	(1.1)
Triglycerides (mg/dl)	98.5	(69.3)	128.1	(92.3)	78.7	(40.6)
Cholesterol (mg/dl)	171.0	(36.9)	173.3	(46.7)	169.4	(29.9)
HDL cholesterol (mg/dl)	53.0	(12.9)	44.2	(6.9)	58.9	(12.6)
LDL cholesterol (mg/dl)	98.7	(27.8)	104.8	(34.2)	94.7	(22.8)
Systolic BP (mmHg)	117.7	(12.4)	129.3	(9.6)	110.7	(7.8)
Diastolic BP (mmHg)	72.0	(7.3)	76.4	(7.5)	69.3	(5.8)
Fatty liver index	24.3	(29.7)	39.1	(37.7)	14.48	(18.0)
Brown adipose tissue						
BAT volume (ml)	72.1	(67.0)	67.8	(74.1)	75.1	(63.9)
BAT SUV peak	10.5	(7.3)	8.2	(6.1)	12.1	(7.8)
BAT mean radiodensity (HU)*	-59.9	(13.1)	-54.4	(11.5)	-57.3	(11.5)

Results are shown as mean (standard deviation). *Sample size for this variable = 24 (9 men, 15 women). BAT: Brown adipose tissue; BP: blood pressure; HDL: high density lipoproteins; HOMA-IR: homeostatic model assessment insulin resistance; HU: Hounsfield units; LDL: low density lipoproteins; SUV: Standardized uptake value; VAT: visceral adipose tissue.

Cold exposure modulates plasma levels of batokines in young human adults

There was an increased in levels of CXCL14 (6.26±2.18 vs 6.84±2.45 ng/ml, Δ=9.3%, P=0.007), GDF15 (290.40±290.39 vs 310.02±102.82 pg/ml, Δ=6.8%, P=0.013), and FGF21 (146.42±84.07 vs 180.15±122.28 pg/ml, Δ=23%, P=0.003) after 2h of cold exposure. Moreover, plasma levels of IL-6 were also upregulated after 1h of cold exposure (6.40±10.49 vs 8.38±12.86 pg/ml, Δ=31%, P=0.048). In contrast, circulating BMP8b was reduced after 2h of cold exposure (367.23±224.83 vs 314.87±216.73 pg/ml, Δ=-14.3%, P=0.022) (**Figure 10**).

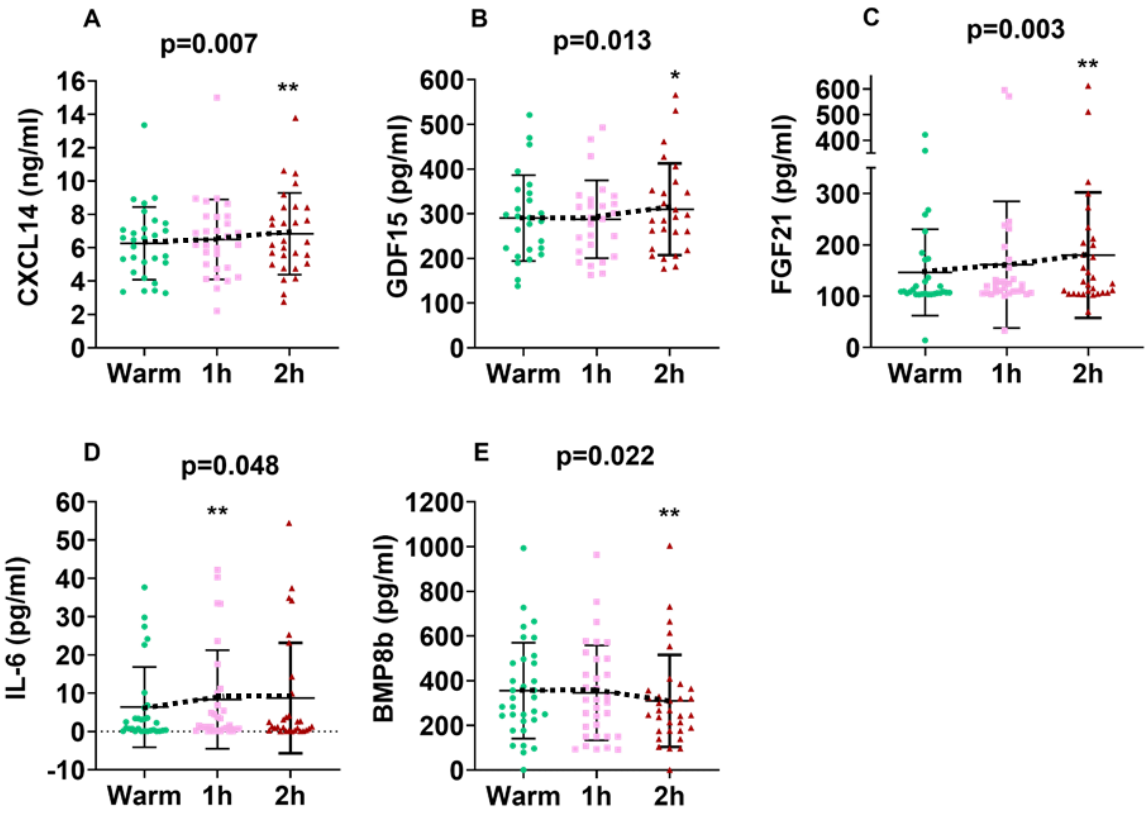


Figure 10. *Effect of a 2-hour personalized cold exposure on the plasma concentration of batokines. The final sample size is n=29 for chemokine ligand 14 (CXCL14), n=26 for growth differentiation factor 15 (GDF15), n=30 for fibroblast growth factor 21 (FGF21), n=24 for Interleukin-6 (IL-6), n=27 for Bone morphogenic protein b8 (BMP8b). Bars represent mean and standard deviation. P from repeated measures analyses of variance (ANOVA). * Significantly different than the warm time point (p<0.05). ** significantly different than the warm time point (p<0.01). FGF21 and IL-6 did not follow a normal distribution, but similar results were observed when performing a Friedman test (non-parametric test) (p=0.001).*

The cold-induced change in FGF21 levels, but not the other batokines, is associated with BAT volume

The cold-induced increase in FGF21 plasma levels after 1h (β=0.389, R^2=0.426, P=0.005) and 2h (β=0.456, R^2=0.307, P=0.001) of cold exposure was positively associated with BAT volume (**Figure 11**), and these results remained after adjusting for the PET-CT scan date, sex, and BMI (**Tables 6-7**). To confirm that these results were independent of warm period plasma levels, we repeated the analyses using fold change instead of the Δ, and the pattern remained, although the strength of association was attenuated (data not shown). In contrast, neither the FGF21 levels during the warm period nor the cold-induced change in its concentration were associated with BAT SUVpeak or radiodensity (**Figure 11**).

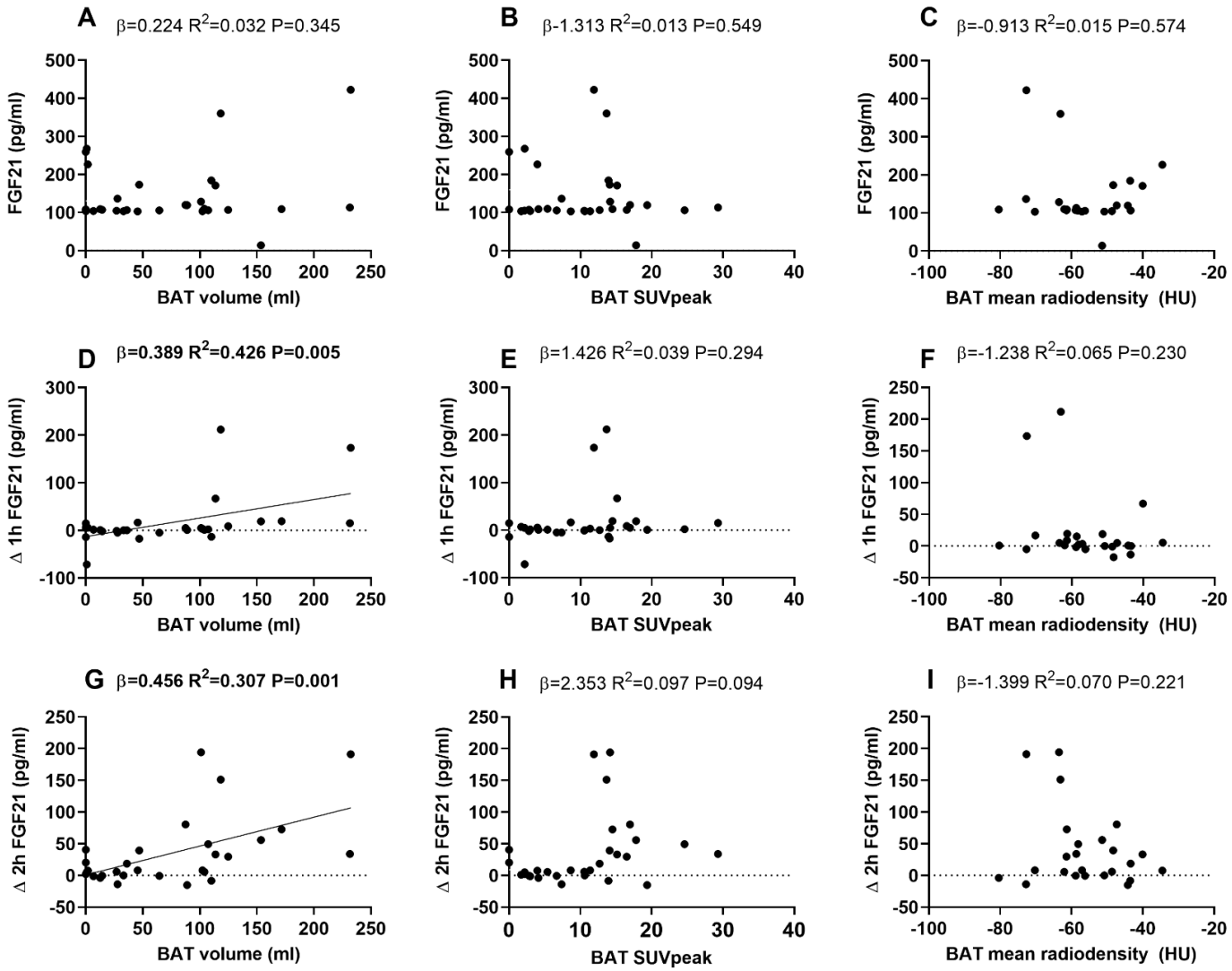


Figure 11. *Associations between the warm and cold-induced changes in fibroblast growth factor 21 (FGF21) circulating concentration and brown adipose tissue (BAT) volume, ^{18}F-Fluoruodeoxyglucose (^{18}F-FDG) uptake and mean radiodensity. Unstandardized β, R^2, and P from simple linear regressions. SUV: Standardized uptake value; HU: Hounsfield units.*

Table 6. *Associations between cold-induced changes in the plasma levels of batokines after one hour of cold exposure and brown adipose tissue (BAT) volume, ^{18}F-Fluorodeoxyglucose uptake and mean radiodensity (n=30*).*

	Δ CXCL14			Δ GDF15			Δ FGF21			Δ Interleukin 6			Δ BMP8b		
	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P
Model 2 (Date PET-CT)															
BAT volume (ml)	<0.001	0.010	0.944	0.036	0.006	0.804	**0.377**	**0.419**	**0.022**	-0.009	0.020	0.481	-0.074	0.003	0.803
BAT SUVpeak	0.033	0.052	0.294	1.206	0.037	0.374	0.444	0.291	0.780	-0.055	0.010	0.622	2.683	0.037	0.345
BAT radiodensity (HU)	0.023	0.094	0.253	1.240	0.135	0.117	-1.353	0.114	0.192	**0.142**	**0.226**	**0.041**	**3.731**	**0.343**	**0.023**
Model 3 (Date PET-CT + Sex)															
BAT volume (ml)	<0.001	0.044	0.989	0.039	0.193	0.778	**0.395**	**0.441**	**0.015**	-0.007	0.128	0.553	-0.043	0.024	0.887
BAT SUVpeak	0.026	0.068	0.433	2.042	0.333	0.137	1.178	0.378	0.479	0.011	0.117	0.926	4.058	0.096	0.187
BAT radiodensity (HU)	0.025	0.122	0.226	1.171	0.226	0.129	-1.558	0.244	0.119	**0.126**	**0.386**	**0.048**	**3.677**	**0.393**	**0.024**
Model 4 (Date PET-CT + BMI)															
BAT volume (ml)	0.001	0.030	0.808	0.052	0.249	0.733	**0.345**	**0.424**	**0.038**	-0.008	0.020	0.503	0.043	0.098	0.884
BAT SUVpeak	0.030	0.060	0.358	1.168	0.264	0.419	0.976	0.335	0.541	-0.063	0.014	0.591	2.164	0.120	0.438
BAT radiodensity (HU)	0.021	0.102	0.323	1.252	0.135	0.131	-0.828	0.293	0.393	**0.158**	**0.258**	**0.031**	**3.670**	**0.345**	**0.031**

Unstandardized β, R^2, and P from simple and linear regressions. Model 2: Model 1 adjusted for the date when the positron emission tomography-computerized tomography (PET-CT) was performed. Model 3: Model 2 additionally adjusted for sex. Model 4: Model 2 additionally adjusted for body mass index (BMI). *24 participants (9 men, 15 women) were included in the BAT radiodensity analyses. BMP8b: Bone morphogenic protein b8; CXCL14: Chemokine ligand 14; FGF21: Fibroblast growth factor 21; GDF15: Growth differentiation factor 15; IL6: Interleukin-6; SUV: Standardized uptake; HU: Hounsfield units.

Table 7. *Associations between cold-induced changes in plasma levels of batokines after two hours of cold exposure and brown adipose tissue (BAT) volume, ^{18}F-Fluorodeoxyglucose uptake and mean radiodensity (n=30*).*

	Δ CXCL14			Δ GDF15			Δ FGF21			Δ Interleukin 6			Δ BMP8b		
	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P
Model 2 (Date PET-CT)															
BAT volume (ml)	0.001	0.002	0.843	8.985	0.074	0.191	**0.573**	**0.539**	**0.001**	-0.009	0.015	0.688	-1.270	0.004	0.911
BAT SUVpeak	0.001	<0.001	0.968	2.890	0.130	0.077	2.671	0.353	0.114	-0.006	0.009	0.978	0.953	0.008	0.734
BAT radiodensity (HU)	-0.002	0.106	0.130	0.450	0.012	0.657	-1.406	0.070	0.222	0.092	0.021	0.515	1.323	0.033	0.520
Model 3 (Date PET-CT + Sex)															
BAT volume (ml)	0.001	0.006	0.830	0.121	0.023	0.504	**0.588**	**0.539**	**0.001**	-0.008	0.030	0.728	0.300	0.109	0.325
BAT SUVpeak	0.005	0.005	0.879	3.310	0.154	0.059	3.461	0.353	0.114	0.044	0.027	0.842	3.033	0.110	0.316
BAT radiodensity (HU)	0.029	0.117	0.154	0.439	0.013	0.673	-1.568	0.140	0.173	0.081	0.043	0.574	1.429	0.271	0.438
Model 4 (Date PET-CT + BMI)															
BAT volume (ml)	0.001	0.002	0.864	0.118	0.022	0.530	**0.572**	**0.550**	**0.001**	-0.003	0.085	0.895	0.635	0.022	0.957
BAT SUVpeak	0.002	0.001	0.949	3.257	0.154	0.060	3.0608	0.402	0.078	-0.076	0.090	0.715	1.512	0.033	0.606
BAT radiodensity (HU)	0.032	0.113	0.127	0.510	0.016	0.630	-1.252	0.083	0.298	0.050	0.086	0.727	1.981	0.111	0.348

*Unstandardized β, R^2, and P from simple and linear regressions. Model 2: Model 1 adjusted for the date when the positron emission tomography-computerized tomography (PET-CT) was performed. Model 3: Model 2 additionally adjusted for sex. Model 4: Model 2 additionally adjusted for body mass index (BMI). *24 participants (9 men, 15 women) were included in the BAT radiodensity analyses. BMP8b: Bone morphogenic protein b8; CXCL14: Chemokine ligand 14; FGF21: Fibroblast growth factor 21; GDF15: Growth differentiation factor 15; IL6: Interleukin-6; SUV: Standardized uptake; HU: Hounsfield units.*

On the other hand, the plasma levels of GDF15 during the warm period were negatively associated with BAT mean radiodensity (β=-3.853, R^2=0.223, P=0.026, **Table 8**) but not BAT volume or ^{18}F-FDG uptake, which persisted after adjusting for the PET-CT scan date, PET-CT scan date + sex, and PET-CT scan + BMI (**Table 9**). This contrasts with the lack of association between the cold-induced changes in GDF15 levels and all BAT related variables (**Table 9**). The changes in plasma levels of IL-6 and BMP8b after 1h of cold exposure were associated with BAT mean radiodensity only when the analyses were adjusted for the PET-CT scan date, sex, or BMI (**Table 6**). In contrast, no associations were observed with the warm and 2h change levels or any other BAT variable (**Table 8**). None of the other batokines were associated with any BAT-related parameter.

Table 8*. Associations between the plasma concentration of batokines before and after 1 and 2 hours of cold exposure and brown adipose tissue (BAT) volume, ^{18}F-Fluorodeoxyglucose uptake and mean radiodensity (n=30).*

	CXCL14 (ng/ml)			GDF15 (pg/ml)			IL-6 (pg/ml)			BMP8b (pg/ml)		
Warm period	**β**	**R^2**	**P**	**β**	**R^2**	**P**	**β**	**R^2**	**P**	**β**	**R^2**	**P**
BAT volume (ml)	0.004	0.016	0.511	0.151	0.009	0.643	0.004	0.001	0.900	0.755	0.052	0.243
BAT SUVpeak	0.043	0.021	0.453	-1.688	0.012	0.589	0.099	0.005	0.717	3.028	0.010	0.612
BAT radiodensity (HU)	0.073	0.127	0.088	**-3.853**	**0.223**	**0.026**	0.378	0.153	0.059	-2.563	0.011	0.649
Δ 1 hour of cold exposure												
BAT volume (ml)	0.001	0.004	0.742	0.014	0.001	0.910	-0.007	0.018	0.480	-0.055	0.002	0.814
BAT SUVpeak	0.031	0.052	0.236	0.860	0.021	0.478	-0.049	0.010	0.599	1.481	0.020	0.486
BAT radiodensity (HU)	0.025	0.069	0.215	1.214	0.118	0.118	0.132	0.152	0.059	3.003	0.154	0.079
Δ 2 hour of cold exposure												
BAT volume (ml)	0.001	0.001	0.844	0.108	0.020	0.487	-0.001	<0.001	0.946	0.194	0.025	0.424
BAT SUVpeak	0.002	<0.001	0.953	2.482	0.116	0.089	0.043	0.002	0.803	1.030	0.008	0.643
BAT radiodensity (HU)	0.029	0.105	0.123	0.440	0.010	0.655	0.089	0.020	0.515	1.134	0.017	0.568

Unstandardized β, R^2, and P from simple regressions. CXCL14: Chemokine ligand 14; GDF15: Growth differentiation factor 15; IL-6: Interleukin-6; BMP8b: Bone morphogenic protein 8b; SUV: Standardized uptake; HU: Hounsfield units.

Table 9. *Associations between plasma levels of batokines of warm period and brown adipose tissue (BAT) volume, ^{18}F-Fluorodeoxyglucose uptake and mean radiodensity (n=30*).*

	CXCL14			GDF15			FGF21			Interleukin 6			BMP8b		
	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P
Model 2 (Date PET-CT)															
BAT volume (ml)	-0.001	0.079	0.916	0.105	0.193	0.775	0.319	0.325	0.265	-0.015	0.035	0.676	-0.216	0.209	0.770
BAT SUVpeak	0.001	0.078	0.990	-2.782	0.199	0.425	-1.828	0.302	0.489	-0.044	0.029	0.894	-7.875	0.251	0.237
BAT radiodensity (HU)	0.069	0.1171	0.110	**-3.912**	**0.239**	**0.027**	-0.950	0.017	0.570	0.369	0.160	0.071	-4.601	0.242	0.373
Model 3 (Date PET-CT + Sex)															
BAT volume (ml)	<0.001	0.180	0.997	0.101	0.193	0.786	0.349	0.337	0.219	-0.012	0.064	0.730	-0.118	0.232	0.876
BAT SUVpeak	0.040	0.192	0.548	-3.794	0.295	0.308	-0.841	0.368	0.762	0.062	0.061	0.857	-6.585	0.256	0.379
BAT radiodensity (HU)	0.060	0.294	0.140	**-3.901**	**0.239**	**0.032**	-1.215	0.107	0.462	0.358	0.170	0.088	-4.550	0.249	0.390
Model 4 (Date PET-CT + BMI)															
BAT volume (ml)	<0.001	0.080	0.953	0.061	0.021	0.876	0.153	0.330	0.525	-0.388	0.070	0.779	-0.099	0.227	0.897
BAT SUVpeak	-0.002	0.079	0.979	-2.476	0.041	0.504	0.020	0.325	0.993	-0.126	0.072	0.707	-9.791	0.291	0.155
BAT radiodensity (HU)	0.069	0.171	0.131	**-3.967**	**0.240**	**0.032**	0.039	0.280	0.979	0.354	0.164	0.100	-6.523	0.326	0.214

Unstandardized β, R^2, and P from simple and linear regressions. Model 2: Model 1 adjusted for the date when the positron emission tomography-computerized tomography (PET-CT) was performed. Model 3: Model 2 additionally adjusted for sex. Model 4: Model 2 additionally adjusted for body mass index (BMI). *24 participants (9 men, 15 women) were included in the BAT radiodensity analyses. BMP8b: Bone morphogenic protein b8; CXCL14: Chemokine ligand 14; FGF21: Fibroblast growth factor 21; GDF15: Growth differentiation factor 15; IL6: Interleukin-6; SUV: Standardized uptake; HU: Hounsfield units.

Association of plasma levels of batokines with body composition and cardiometabolic risk factors.

The associations of the levels of batokines with body composition and cardiometabolic risk factors are shown in the supplementary material (**Table 10** and **12**). Levels of FGF21 during the warm period was positively associated with BMI, WC, lean mass, fat mass, BAT mass, circulating glucose and insulin levels, HOMA-IR, triglycerides, total cholesterol, LDL-C, systolic and diastolic blood pressure (BP) and fatty liver index (**Table 10**). Despite these associations were not replicated with the cold-induced change in FGF21 levels 1h after cold exposure (**Table 11**), we observed a positive association between the cold-induced change in FGF21 concentration after 2h of cold exposure and insulin, HOMA-IR, triacylglycerols, total cholesterol, and LDL-C (**Table 12**). However, these associations disappeared after adjusting for the FGF21 concentration during the warm period. On the other hand, levels of CXCL14 during the warm period were positively associated with fasting insulinemia (β=1.606, R^2=0.138, P=0.048), LDL-C (β=4.805, R^2=0.139, P=0.047) and systolic BP (β=2.467, R^2=0.198, P=0.018) (**Table 10)**. The plasma levels of GDF15 during the warm period were also positively associated with insulin levels (β=0.048, R^2=0.272, P=0.006) and HOMA-IR (β=0.013, R^2=0.248, P=0.010) (**Table 10)**.

Table 10. *Associations between the plasma levels of batokines during the warm period and body composition, and cardiometabolic risk factors (n=30).*

	CXCL14 (ng/ml)			GDF15 (pg/ml)			FGF21 (pg/ml)			IL6 (pg/ml)			BMP8b (pg/ml)		
	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P
BMI (kg/m^2)	0.046	<0.001	0.920	0.006	0.014	0.559	**0.036**	**0.358**	**<0.001**	-0.141	0.091	0.152	-0.003	0.019	0.491
WC (cm)	0.915	0.018	0.493	0.019	0.015	0.557	**0.107**	**0.369**	**<0.001**	-0.069	0.003	0.811	0.003	0.002	0.845
Lean mass (kg)	0.828	0.030	0.371	0.014	0.017	0.530	**0.060**	**0.238**	**0.006***	-0.001	<0.001	0.994	0.008	0.023	0.449
Fat mass (kg)	0.141	0.001	0.873	0.019	0.036	0.352	**0.067**	**0.331**	**0.001**	-0.278	0.091	0.151	-0.004	0.007	0.675
Fat mass %	-0.575	0.022	0.443	0.010	0.013	0.579	0.027	0.075	0.143	-0.270	0.121	0.096	-0.008	0.043	0.299
VAT mass (g)	14.643	0.022	0.439	0.374	0.029	0.406	**1.561**	**0.374**	**<0.001**	-3.810	0.035	0.379	-0.091	0.008	0.660
Glucose (mg/dl)	1.007	0.066	0.176	0.018	0.047	0.285	**0.064**	**0.414**	**<0.001**	0.020	0.001	0.906	0.010	0.061	0.214
Insulin (µUl/ml)	**1.606**	**0.138**	**0.047**	**0.048**	**0.272**	**0.006**	**0.087**	**0.614**	**<0.001**	-0.122	0.018	0.530	0.012	0.078	0.157
HOMA-IR	0.423	0.123	0.062	**0.013**	**0.248**	**0.010**	**0.024**	**0.624**	**<0.001**	-0.031	0.015	0.574	0.003	0.078	0.158
Triglycerides (mg/dl)	10.315	0.103	0.090	0.144	0.042	0.317	**0.585**	**0.504**	**<0.001**	-0.456	0.005	0.754	0.024	0.005	0.716
Total cholesterol (mg/dl)	4.898	0.082	0.131	0.056	0.019	0.502	**0.249**	**0.323**	**0.001**	-0.778	0.050	0.293	-0.005	0.001	0.884
HDL-C (mg/dl)	-1.619	0.073	0.158	-0.007	0.004	0.763	-0.037	0.059	0.198	-0.293	0.054	0.275	<0.001	<0.001	0.970
LDL-C (mg/dl)	**4.805**	**0.139**	**0.047**	0.048	0.025	0.442	**0.191**	**0.332**	**0.001**	-0.414	0.025	0.458	-0.005	0.002	0.838
Systolic BP (mm Hg)	**2.467**	**0.198**	**0.018**	0.012	0.010	0.642	**0.078**	**0.222**	**0.010***	-0.018	<0.001	0.940	0.004	0.005	0.737
Diastolic BP (mm Hg)	0.690	0.048	0.262	0.001	<0.001	0.957	**0.050**	**0.267**	**0.004**	-0.022	0.001	0.880	-0.005	0.022	0.471
Fatty liver index	2.526	0.034	0.341	0.034	0.013	0.583	**0.251**	**0.506**	**<0.001**	-0.287	0.010	0.632	-0.016	0.013	0.574

Unstandardized β, R^2, and P from simple and linear regressions. * the association disappeared when the variable was log10 transformed. BMP8b: Bone morphogenic protein b8; BMI: Body mass index; BP: blood pressure; CXCL14: Chemokine ligand 14; FGF21: Fibroblast growth factor 21; GDF15: Growth differentiation factor 15; HDL: high density lipoproteins; HOMA-IR: homeostatic model assessment insulin resistance; IL6: Interleukin-6; LDL: low density lipoproteins; VAT: visceral adipose tissue; WC: Waist circumference;

Table 11. *Associations between cold-induced changes in plasma concentration of batokines after one hour of cold exposure and body composition and cardiovascular risk factors (n=30).*

	Δ CXCL14 (ng/ml)			Δ GDF15 (pg/ml)			Δ FGF21 (pg/ml)			Δ IL6 (pg/ml)			Δ BMP8b (pg/ml)		
	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P
BMI (kg/m^2)	-0.360	0.005	0.717	-0.010	0.006	0.702	0.025	0.068	0.163	-0.151	0.012	0.610	-0.020	0.097	0.122
WC (cm)	-0.666	0.002	0.819	-0.012	0.001	0.887	0.095	0.115	0.067	0.208	0.003	0.806	-0.043	0.050	0.270
Lean mass (kg)	-3.014	0.084	0.128	0.032	0.015	0.557	0.065	0.108	0.076	0.494	0.032	0.400	-0.006	0.002	0.826
Fat mass (kg)	-0.122	<0.001	0.949	-0.033	0.016	0.539	0.065	0.119	0.062	-0.304	0.013	0.602	-0.043	0.108	0.101
Fat mass %	1.463	0.030	0.368	-0.063	0.077	0.171	0.023	0.021	0.443	-0.635	0.077	0.189	-0.041	0.129	0.072
VAT mass (g)	-21.514	0.013	0.552	-0.340	0.004	0.770	1.072	0.069	0.161	3.901	0.004	0.761	-0.530	0.037	0.347
Glucose (mg/dl)	-1.185	0.019	0.472	0.012	0.003	0.781	0.033	0.042	0.276	0.230	0.010	0.643	-0.037	0.111	0.096
Insulin (µUl/ml)	1.239	0.017	0.495	0.014	0.003	0.774	0.053	0.091	0.105	-0.187	0.005	0.743	-0.049	0.149	0.051
HOMA-IR	0.243	0.009	0.633	0.005	0.005	0.731	0.016	0.104	0.082	-0.042	0.003	0.793	**-0.014**	**0.161**	**0.043#**
Triglycerides (mg/dl)	3.478	0.002	0.798	0.249	0.019	0.505	0.309	0.055	0.212	2.135	0.012	0.618	-0.276	0.088	0.142
Total cholesterol (mg/dl)	3.393	0.008	0.637	0.016	<0.001	0.940	0.185	0.069	0.160	0.067	<0.001	0.976	**-0.194**	**0.160**	**0.043#**
HDL-C (mg/dl)	-1.858	0.020	0.462	-0.062	0.042	0.314	0.015	0.004	0.749	-0.571	0.024	0.472	-0.005	0.003	0.808
LDL-C (mg/dl)	4.417	0.025	0.415	0.037	0.002	0.822	0.141	0.070	0.156	0.180	0.001	0.913	**-0.148**	**0.158**	**0.044#**
Systolic BP (mm Hg)	-2.812	0.055	0.231	0.076	0.058	0.247	0.085	0.067	0.174	1.036	0.101	0.140	<0.001	<0.001	0.999
Diastolic BP (mm Hg)	-2.374	0.121	0.069	0.048	0.065	0.217	0.035	0.034	0.340	0.572	0.084	0.179	0.010	0.013	0.584
Fatty liver index	0.005	<0.001	0.999	0.063	0.007	0.689	0.117	0.043	0.272	0.853	0.010	0.635	-0.114	0.086	0.146

Unstandardized β, R^2, and P from simple and linear regressions. # the association disappeared after adjusting for BMI. BMP8b: Bone morphogenic protein b8; BMI: Body mass index; BP: blood pressure; CXCL14: Chemokine ligand 14; FGF21: Fibroblast growth factor 21; GDF15: Growth differentiation factor 15; HDL: high density lipoproteins; HOMA-IR: homeostatic model assessment insulin resistance; IL6: Interleukin-6; LDL: low density lipoproteins; VAT: visceral adipose tissue; WC: Waist circumference;

Table 12. *Associations between cold-induced changes in plasma concentration of batokines after two hours of cold exposure and body composition and cardiovascular risk factors (n=30).*

	Δ CXCL14 (ng/ml)			Δ GDF15 (pg/ml)			Δ FGF21 (pg/ml)			Δ IL6 (pg/ml)			Δ BMP8b (pg/ml)		
	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P
BMI (kg/m^2)	0.540	0.012	0.623	0.006	0.003	0.799	0.011	0.015	0.517	-0.249	0.119	0.098	0.009	0.021	0.475
WC (cm)	-0.666	0.002	0.819	0.038	0.013	0.576	0.047	0.031	0.353	-0.464	0.051	0.289	0.027	0.022	0.457
Lean mass (kg)	-3.014	0.084	0.128	-0.005	<0.001	0.921	0.029	0.025	0.409	-0.039	0.001	0.898	0.015	0.013	0.565
Fat mass (kg)	-0.122	<0.001	0.949	0.039	0.033	0.375	0.042	0.054	0.215	-0.514	0.132	0.081	0.013	0.011	0.600
Fat mass %	1.463	0.030	0.368	0.028	0.023	0.458	0.023	0.023	0.427	**-0.490**	**0.168**	**0.046***	0.003	0.001	0.897
VAT mass (g)	-24.514	0.013	0.552	0.532	0.014	0.572	0.663	0.029	0.369	-8.070	0.067	0.221	0.671	0.065	0.199
Glucose (mg/dl)	-1.185	0.019	0.472	0.002	<0.001	0.952	0.052	0.115	0.067	-0.093	0.006	0.719	-0.014	0.019	0.497
Insulin (μUl/ml)	1.239	0.017	0.495	0.041	0.045	0.296	**0.062**	**0.136**	**0.045***	-0.360	0.067	0.221	-0.005	0.002	0.832
HOMA-IR	0.243	0.009	0.633	0.011	0.038	0.339	**0.018**	**0.155**	**0.032***	-0.089	0.053	0.280	-0.002	0.005	0.718
Triglycerides (mg/dl)	3.478	0.002	0.798	0.265	0.032	0.378	**0.467**	**0.138**	**0.043***	-3.138	0.091	0.151	0.038	0.002	0.823
Total cholesterol (mg/dl)	3.393	0.008	0.637	-0.152	0.032	0.381	**0.313**	**0.219**	**0.009***	-1.843	0.120	0.098	0.064	0.021	0.468
HDL-C (mg/dl)	-1.858	0.020	0.462	-0.028	0.014	0.570	0.010	0.002	0.830	-0.146	0.006	0.726	-0.019	0.042	0.306
LDL-C (mg/dl)	4.417	0.025	0.415	0.139	0.047	0.287	**0.237**	**0.221**	**0.009***	-1.110	0.077	0.189	0.070	0.043	0.302
Systolic BP (mm Hg)	-2.812	0.055	0.231	0.062	0.058	0.246	0.024	0.010	0.603	-0.197	0.014	0.597	0.007	0.007	0.694
Diastolic BP (mm Hg)	-2.374	0.121	0.069	0.009	0.003	0.788	0.006	0.002	0.821	-0.167	0.026	0.458	0.017	0.013	0.585
Fatty liver index	7.018	0.053	0.227	0.088	0.020	0.490	0.110	0.042	0.280	-1.250	0.082	0.175	0.067	0.033	0.362

Unstandardized β, R^2, and P from simple linear regressions. * The association disappeared after adjusting for the baseline level of the batokine. BMP8b: Bone morphogenic protein b8; BMI: Body mass index; BP: blood pressure; CXCL14: Chemokine ligand 14; FGF21: Fibroblast growth factor 21; GDF15: Growth differentiation factor 15; HDL: high density lipoproteins; HOMA-IR: homeostatic model assessment insulin resistance; IL6: Interleukin-6; LDL: low density lipoproteins; VAT: visceral adipose tissue; WC: Waist circumference;

Discussion

The results of this study show that the levels of the five potential batokines (CXCL14, GDF15, FGF21, IL-6, and BMP8b) are modified by a 2h cold exposure in young human adults. Moreover, the cold-induced change in FGF21 levels was positively associated with BAT volume. Overall, these results suggest that these 5 batokines may be part of the endocrine response to cold exposure in humans, with FGF21 possibly being secreted by BAT. However, more studies in humans are needed to clarify the role of BAT in the release of FGF21 and the rest of batokines.

We first investigated whether the levels of the 5 potential batokines (CXCL14, GDF15, FGF21, IL-6 and BMP8b) were modulated by a cooling protocol able to activate BAT in humans. We observed a cold-induced increase in the concentration of CXCL14, GDF15, IL-6, and FGF21. We have previously shown that circulating levels of CXCL14 are increased in response to cold exposure in mice, and that BAT secretion is partially responsible of this increase in these animals [35]. Importantly, this increase in CXCL14 was associated with improved glucose homeostasis, greater BAT activity and WAT browning through the recruitment of alternatively activated, non-inflammatory macrophages [35]. In the present human study, the cold-induced increase in plasma levels of CXCL14 was not associated with BAT volume, ^{18}F-FDG uptake or radiodensity. In contrast with that, García-Beltran et al. [36] reported that CXCL14 levels correlated positively with the area of active cervical BAT, although only in one year-old girls [36], which concurred with high CXCL14 gene expression levels in neonatal BAT [36]. Despite the age of the participants is an obvious key difference in our study, it should also be noted that García-Beltran et al. [36] used infrared thermography instead of PET-CT to evaluate BAT activity (and the 2 methods have a very limited agreement [37,38]) [36]. On the other hand, GDF15, an hormone that is currently being intensively investigated due to its potential role in appetite regulation, glucose homeostasis and lipolysis [39,40], seems to be secreted by murine brown adipocytes in response to cold exposure [41]. Here we observed a cold-induced increase in GDF15 levels, which would be compatible with a BAT secretion in humans. However, as for CXCL14, we did not observe consistent associations between the cold-induced increase in GDF15 concentration and any BAT-related variable. Finally, our analyses also showed an increase in IL-6 plasma levels after 1h of cold exposure. It is well known that brown adipocytes secrete IL-6 [22], orchestrating a BAT-to-liver communication [42]. IL-6 can promote WAT browning and BAT recruitment by activation of M2-type macrophage [43–45], and seems to be a key mediator of the benefits associated with BAT transplantation in murine models [18]. However, we did not find consistent associations between the cold-induced changes in IL-6 levels and BAT volume, ^{18}F-FDG uptake or radiodensity. It should be noted that skeletal muscle is a well-known secretor of IL-6, and thus, cold-induced muscle activity (and IL-6 release) might be confounding our results, even if our cooling protocol precluded shivering [46]. Thus, future

studies are necessary to ascertain if the cold-induced increases observed in this study are indeed due to the increased BAT secretion of these molecules.

FGF21 was one of the first described batokines [21] and has been shown to target several tissues and organs such as adipose tissue, the heart and the liver [47–49]. In our study, we observed an increase in FGF21 levels in response to cold exposure with this increase being robustly associated with BAT volume. This finding concurs with a previous study showing an increase in plasma levels of FGF21 after 12h of exposure to mild cold (19ºC) air [50]. The same research group [51], and others [52], observed that this increase was more pronounced in BAT-positive than in BAT-negative participants. Moreover, another study found a positive association between the levels of FGF21 and BAT glucose uptake [53]. Overall, our results and previous studies suggest that FGF21 may be secreted by human BAT to an extent that may affect the systemic circulation. However, it is known that BAT is not the only source of FGF21 after cold. Reports using various cold-induced experimental setting in rodent models claimed distinct roles of liver and BAT as contributing to systemic FGF21 levels after cold exposure [54–56]. Therefore, further studies in humans are required to analyze the tissue(s) responsible for the increase of FGF21 in the circulation and its metabolic role. FGF21 modulates insulin sensitivity [55], protect the liver against steatosis [57,58], and exerts cardioprotective actions in mouse models of hypertension [59], cardiac hypertrophy [59,60] and ischemia [61]. Therefore, it might seem paradoxical that we observed a positive association between the plasma levels of FGF21 during the warm period and several markers of adiposity and cardiometabolic risk, including BMI, WC, VAT mass, insulin, HOMA-IR, and cholesterol. Consistent with the results observed in our study, others have also reported an increased FGF21 levels in obese subjects and a positive correlation with adiposity, insulin and triglycerides [62–64]. This paradox between the beneficial effects of FGF21 on metabolism and the observed augmented circulating levels in human adults with overweight and obesity might be explained by an "FGF21-resistant" state in these subjects [65]. Further studies with a larger sample are needed to clarify not only the role of BAT in FGF21 secretion, but also its impact on human cardiometabolic health.

In contrast with the changes observed in the levels of CXCL14, GDF15, IL-6 and FGF21, we observed a cold-induced decrease in BMP8b. BMP8b seems to be secreted by brown adipocytes in mice and *in vitro* studies [23,66]. However, its role on BAT activity is controversial [67,68]. Whereas Urisarri et al. [68] observed a positive association between BMP8b circulating levels and BAT thermogenic response, measured with infrared thermography, after cold exposure in neonates , Garcia-Beltran et al. [67] did not observed any correlation in 1 year-old infants, despite reporting a high BMP8b expression levels relative to WAT in neonates . The apparent discordance between previous studies and our results might be due to several reasons. Firstly, most of BAT BMP8b might exert autocrine and paracrine roles [66], with negligible contributions to the circulatory pool,

at least, in human adults. Second, longer cooling protocol may yield different results, having into account that 2h cooling period is likely too short to reflect changes in protein expression [69,70]. Third, changes in the levels of BMP8b cannot be interpreted exclusively as a function of BAT secretion, as other tissues also express this protein (https://www.proteinatlas.org/ENSG00000116985-BMP8B/tissue). Either a reduction of BMP8b secretion by other organs or an increase degradation would also explain why its blood levels are reduced despite a presumable increase in BAT secretion. Finally, the distinct methods used for assessing BAT (PET-CT vs. infrared thermography) are not comparable and may be responsible of the observed discrepancies among studies.

In this study, we aimed to investigate the modulation of selected batokines after BAT thermogenic stimulation in humans, which may be responsible for a healthier cardiometabolic status [71]. However, there is an important gap in the knowledge of the secretory role of human BAT *in vivo* [72]. We hypothesized that if BAT secretes a molecule to a sufficient extent to impact the systemic circulating pool, the concentration of this molecule should increase after BAT by cold exposure as well as correlate with indicators of BAT volume or activity. It should be noted that the confirmation of these hypotheses would be highly suggestive of a physiologically relevant secretion of this molecule by BAT. However, the refutation of the hypotheses does not disprove this possibility. Firstly, it should be considered that despite BAT is unequivocally able to secrete these molecules [13], no batokine identified to date is exclusively secreted by BAT, such as FGF21 [54]. Secondly, it should be considered that despite the static ^{18}F-FDG PET-CT scan is the most widely used technique for assessing BAT volume and function [6], it presents several limitations for assessing BAT metabolic activity [73,74]. In fact, raising insulin plasma concentration induces BAT ^{18}F-FDG uptake without a parallel increase in the tissue blow flow or metabolic activity [75], which illustrates how BAT ^{18}F-FDG uptake and thermogenic activity can be dissociated. Moreover, even if the ^{18}F-FDG PET-CT provided an accurate assessment of BAT thermogenic activity, it should not be assumed that the tissue endocrine activity is necessarily proportional to its thermogenic activity. Finally, the 2h cooling protocol may not be long enough to elicit a significant secretion of some batokines, which would be expectable if such batokines secretion depends on protein synthesis [70]. Nonetheless, even if testing the two proposed hypothesis cannot rule out that human BAT significantly secretes these molecules, confirming the two hypotheses would be highly suggestive of a relevant contribution of BAT secretion to their circulating pool.

Besides the study limitations mentioned before, it should be considered that despite the anatomical region analysed by the PET-CT scan represents the main BAT deposit, there are others excluded (e.g. suprarenal BAT depots) [6]. Moreover, the use of other radiotracers (e.g., [^{15}O]-O_2 or ^{11}C-acetate) or imaging methods (magnetic resonance, dynamic PET acquisition) might be more adequate to assess BAT function than the static ^{18}F-FDG PET-CT performed in this study.

In summary, we found that a 2h personalized cold exposure increases the plasma levels of CXCL14, GDF15, FGF21 and IL-6 in young adults. The observed association between the cold-induced increase in FGF21 and BAT volume suggests that human BAT might secrete this molecule to an extent able to impact to the circulating pool, although more studies are needed to confirm this hypothesis.

CHAPTER 2. Exercise as activator of human BAT

2.1. "Endocrine Mechanisms Connecting Exercise to Brown Adipose Tissue Metabolism: a Human Perspective"

BROWN ADIPOSE TISSUE AND EXERCISE

Exercise increases both energy expenditure and heat production, and it could therefore be expected to downregulate BAT activity and WAT browning[172]. However, although the effect of exercise on classic BAT remains controversial[173], an exercise-induced WAT browning has been consistently reported in rodents[174–176]. Exercise elicits a myriad of endocrine signals that are known to regulate BAT activity and/or WAT browning (**Figure 12**), all of which are reviewed in this section addressing both proteins and metabolites hormones as mediators of endocrine signaling. This section is based on the studied performed by Mendez-Gutierrez et al (2020)[105].

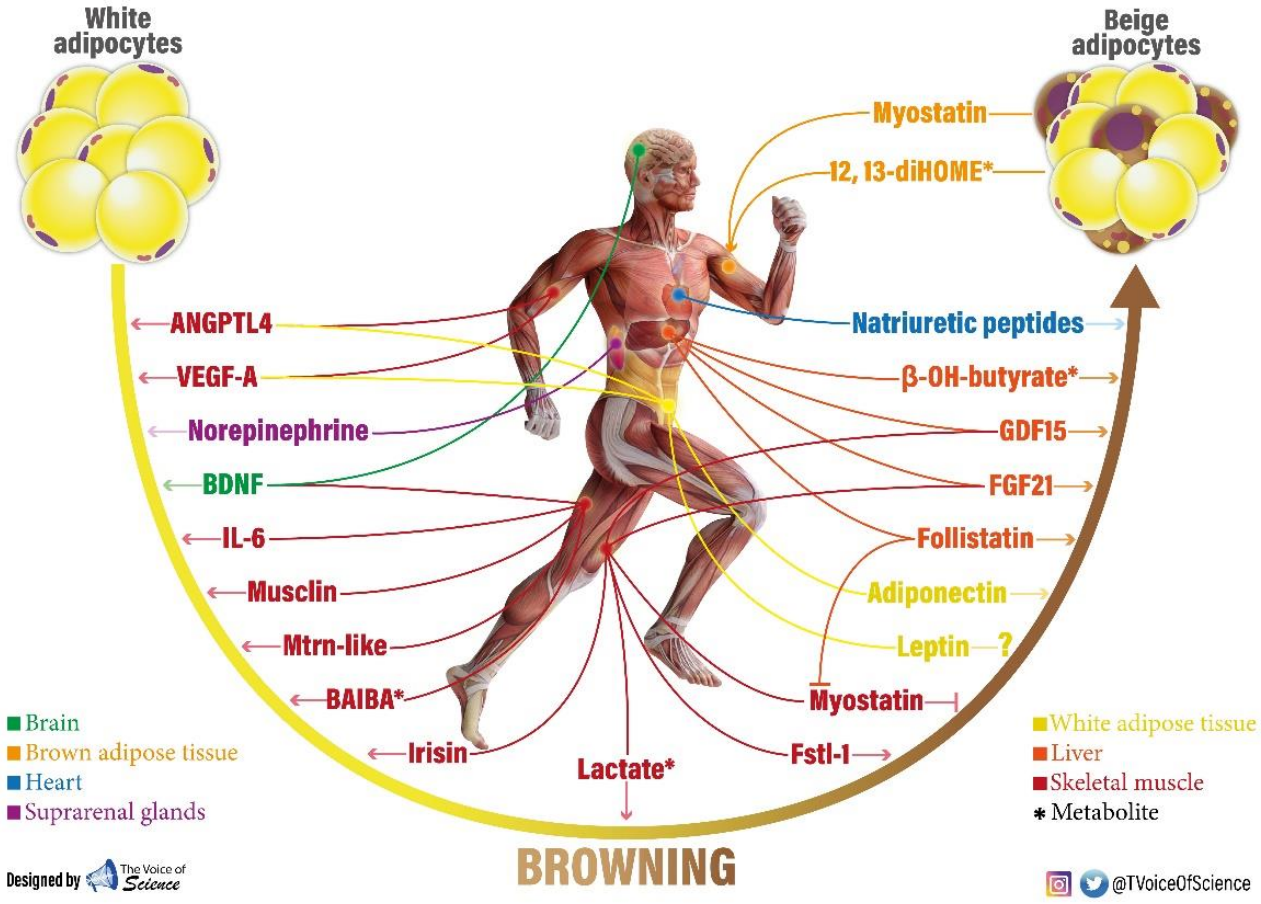


Figure 12. Endocrine mechanisms connecting exercise to brown adipose tissue (BAT) metabolism and white adipose tissue (WAT) browning in humans. Several molecules with capacity to regulate BAT metabolism and/or WAT browning, including protein hormones and metabolites, are secreted during exercise. The brown and beige adipocytes also secrete signalling factors that can influence skeletal muscle metabolism during exercise. The represented secreting tissue is speculative for most of the molecules. The evidence supporting the information depicted in the figure mainly comes from animal studies, and still need to be confirmed in humans. ANGPTL4: Angiopoietin-like 4; Baiba: Beta aminobutyric acid; BDNF: Brain derived neurotrophic factor; β-OH-butyrate; GDF15: Growth differentiation factor 15; FGF21: Fibroblast

growth factor 21; Fstl-1: Follistatin protein like 1; Mtrn-like: Meteorin like; VEGF: Vascular endothelial growth factor A; 12,13-diHOME: 12,13-dihydroxy-9Z-octadecenoic acid (12,13-diHOME). Mendez-Gutierrez et al. Endocrine Mechanisms Connecting Exercise to Brown Adipose Tissue Metabolism: a Human Perspective. Curr Diab Rep. 2020 Jul 28;20(9):40.

PROTEIN HORMONES

Norepinephrine

As it was explained in the General Introduction section, the SNS is the most relevant BAT regulator. Upon cold exposure, released norepinephrine in BAT binds to the brown adipocytes β-adrenergic receptors and activates BAT thermogenesis[14]. Norepinephrine also stimulates lipolysis in adipose tissues[177]. It is well known that adrenal gland produces the increase of norepinephrine circulating levels up to 20-fold[178] in humans after acute exercise (both aerobic and resistance) (**Table 13**). Therefore, although the SNS-dependent activation of BAT is mainly driven by local nerve release of this protein[14], it is still possible that the exercise-induced norepinephrine plasma levels during exercise contributes to BAT activation. Human studies need to be developed to investigate whether such increased norepinephrine levels after exercise modulates BAT function. As well, *in vitro* and *in vivo* studies are required to determine if human BAT is one of the targets of norepinephrine released after exercise.

Atrial Natriuretic Peptide (ANP)

The main function of the heart-secreted natriuretic peptides (NPs) is to regulate blood pressure by modulating diuresis, natriuresis and vasodilatation[179,180]. NPs are also involved in lipolysis induction in WAT[181,182] and fat oxidation in human skeletal muscle[183]. Moreover, NPs promotes energy dissipation in BAT and WAT browning, increasing the expression of *UCP1* and peroxisome proliferator-activated receptor gamma coactivator 1-α (*PGC-1α*)[184]. Exercise stimulates the cardiac muscle, which in turn activate the secretion of atrial natriuretic peptide (ANP)[185]. ANP is a short-life molecule able to induce WAT browning *in vitro*[184,186]. Several studies have reported an increase in ANP circulating levels after both acute moderate and high-intensity endurance exercise in different populations[187–190] (**Table 13**). Due to the scarce scientific evidence, there is still a gap regarding the involvement of exercise in the modulation of circulating levels of human ANP, the effect of different exercise stimuli (i.e. acute, chronic and different types of intensity) and whether this molecule is able to impact BAT in humans.

Irisin

PGC-1α is one of the master transcription factors upregulated by exercise in skeletal muscle. PGC-1α activity increases the expression of the fibronectin type III domain containing 5 (FNDC5) protein. FNDC5, after cleavage, is secreted into the bloodstream as irisin, which, at least in mice, binds to the surface of adipocytes inducing the expression of UCP1 and promoting WAT browning[191–193].

Several human studies have shown an increase in *FDNC5* gene expression in skeletal muscle and circulating serum irisin after acute exercise (**Table 13**). For instance, a 50-minute cycling bout at 80% of maximum oxygen consumption (VO_2max) was able to increase circulating irisin 10 minutes after exercise in both trained and untrained healthy adults[194]. The intensity of exercise may play an important role in the stimulation of irisin secretion[195]. Nonetheless, it should be considered that there are important between-studies inconsistencies related to commercial methods used to detect irisin[196–198]. Moreover, the capacity and specificity of commercially available methods for human irisin detection has been questioned, and thus, important doubts remain regarding the role of irisin in humans and its regulation by exercise[197]. Since the scientific literature is controversial regarding this molecule, more studies are needed to clarify firstly, the presence of circulating irisin in humans and, secondly, the effect of different type of exercise on its levels as well as its action on BAT activation.

Fibroblast growth 21 (FGF21)

Fibroblast growth 21 (FGF21) is one of the endocrine members of the fibroblast growth factor family. It is mainly expressed by the liver, but also secreted by other tissues such as the thymus, WAT, skeletal muscle[199,200] and BAT[138,201]. Indeed, the release of FGF21 is increased in murine brown adipocytes by thermogenic activation[136,137]. FGF21 induces WAT browning through activation of PGC-1α[202,203]. In BAT, FGF21 can act in an autocrine, paracrine and endocrine manner inducing UCP1 expression and BAT thermogenesis[204]. Interestingly, a positive association between circulating FGF21 and BAT volume has been reported in healthy men[140].

Several studies have reported exercise-induced increases in human FGF21 circulating levels, especially during recovery (**Table 13**). Slusher et al (2015) reported an increase in FGF21 plasma levels after exercise in obese and normal-weight subjects, being greater in normal-weight participants[205]. FGF21 circulating levels stay increased up to 6 hours after exercise cessation in normal-weight and overweight/obese men[206]. Moreover, a recent study suggested an exercise intensity-dependent FGF21 secretion[207]. Camperros et al (2020) also reported an increase in circulating FGF21 in runners after a marathon race, returning to baseline levels after 48 hours[208]. Given that circulating levels of FGF21 are increased after exercise, and also this molecule seems to have a relevant role in BAT function, it seems plausible that exercise could activate this tissue through the release of this protein as an endocrine factor. Thus, more studies are needed to

elucidate whether FGF21, studied as an exerkine released after exercise, is able to target human BAT and lead its activation. It would also be interesting to study the effect of different types of exercise stimuli on the levels of the molecule, including acute and chronic exercise.

Interleuquin-6 (IL-6)

Interleuquin-*6* (IL-6) is mainly produced in adipose tissue and skeletal muscle by immune and non-immune cells[209]. In WAT, IL-6 can activate eosinophils to produce interleuquin-4 (IL-4), which induces macrophages to acquire a M2 phenotype and in turn promotes WAT browning by local norepinephrine release[210,211]. Interestingly, it has been shown that the effect of BAT transplantation is not present when the donor was a IL-6 knock-out mouse[145].

It is widely known that acute exercise increases circulating IL-6 up to 100-fold[212] (**Table 13**). Exercise intensity and duration, the form of muscular contraction (eccentric or concentric) and muscle damage are the main mechanisms that mediate the IL-6 response to acute exercise[213]. Despite the large number of studies analyzing the effect of exercise on circulating IL-6 levels, there are no studies to date aimed at determining whether exercise is able to activate human BAT through the release of IL-6.

Meteorin-like protein

The expression in skeletal muscle of a splice form of the gene encoding PGC-1α, termed PGC-1α4, stimulates the synthesis and secretion of a protein called meteorin-like. Upon binding its receptor in adipose tissue, meteorin-like promotes an eosinophil dependent activation of M2 macrophages, secreting IL-4 and IL-13, which in turn induces WAT browning and the expression of genes encoding the thermogenic and mitochondrial program, by means of norepinephrine release[210,214]. Indeed, the *in vitro* administration of an anti-meteorin-like antibody partially prevented cold-induced WAT browning[215]. Meteorin-like is not only produced by skeletal muscle but also by brown and beige adipocytes in response to cold[216].

In a seminal study, Rao et al (2014) showed that meteorin-like mRNA expression is induced in murine skeletal muscle after a resistance exercise session[210]. This overexpression concurred with increased levels of circulating meteorin-like, which remained elevated 24 hours after the exercise session. Importantly, Saghebjoo et al (2018) observed an increase in meteorin-like levels after a session of moderate endurance exercise in young women[211] (**Table 13**).

It would be intriguing to design studies in mice and humans that could reveal the effect of different exercise stimuli on circulating levels of meteorin-like in humans. Furthermore, since the molecule appears to be involved in BAT function *in vitro*, it remains a challenge to find whether this molecule exerts a positive effect on human BAT activation after being released *in*

vivo after exercise.

Musclin

Firstly reported by Nishizawa et al (2004)[217], musclin is a peptide produced by skeletal muscle that can be found in bloodstream[36]. Musclin shares some structural similarities with natriuretic peptides, and consequently, can bind to some common receptors[218]. Musclin promotes mitochondrial biogenesis in skeletal muscle[218]. Moreover, since musclin works as a peroxisome proliferator-activated receptor γ (PPARγ) agonist, it has been suggested to play a role in the browning process[36]. Although it seems that musclin is secreted in response to exercise in murine models[218], yet, whether it is also the case in humans remains to be elucidated. Indeed, there is only one study reporting a non-significant reduction with a small effect size of circulating musclin levels after a 12-week treadmill program in 29 human adults, so further research is required.

Growth differentiation factor-15 (GDF15)

Growth differentiation factor-15 (GDF15) is a protein belonging to the transforming growth factor-β (TGF-β) superfamily, whose receptor is mainly expressed in the brain and in WAT[129,219]. Although the major source of circulating GDF15 is the liver, it is also expressed, among others, in skeletal muscle, WAT and BAT[220–222]. GDF15 is released by brown and beige adipocytes in response to thermogenic activity, targeting BAT macrophages and downregulating local inflammation[130, 208].

Several studies have reported that exercise increases GDF15 circulating levels after a moderate and a high-intensity session in human adults, both endurance and resistance exercise[208,223–225]. Noteworthy, circulating levels of GDF15 are even more elevated during recovery in trained populations[223,225]. Interestingly, Conte et al (2020) observed that circulating GDF15 was higher in sedentary participants comparing with their counterparts, whereas the protein plasma levels after a strenuous physical activity suffered a dramatic increase in experimented cyclists[226].

Due the involvement of GDF15 in BAT function, skeletal muscle activation and metabolic effects (e.g. appetite or fat mass[227]) it would be relevant to stablish whether exercise, indeed, exerts a positive role on surrounding levels of GDF15 and, as a consequence, human BAT would be activated contributing to metabolich health.

Myostatin

Growth differentiation factor-8, also known as myostatin, is another member of the TGF-β superfamily, which was described to be a myokine early in the 1990s[228]. Myostatin´s main function is the inhibition of muscle growth, and consequently, its suppression dramatically stimulates muscle growth[229]. Myostatin loss of function not only results in muscle hypertrophy, but also in a decreased fat accumulatio[230] and WAT browning in mice[231,232]. The induction of WAT browning by myostatin inhibition is triggered by the activation of the AMPK enzyme and the subsequent induction of PGC-1α and FNDC5[233]. Therefore, myostatin seems to play an important role as WAT browning inhibitor. Moreover, BAT-muscle connection through myostatin could be bidirectional, with BAT influencing muscle function by secreting myostatin[95].

Acute and chronic exercise modifies myostatin expression and circulating levels, although this effect seems to be dependent on the type and intensity of exercise[234–237] (**Table 13**). Both acute and chronic trainings decrease myostatin circulating levels in humans[235,238–240], and this effect seems to last up to 24 hours after a resistance training session[235]. Importantly, the myostatin effect on BAT represents a proof of concept that exercise induces the secretion of not only pro-browning agents, but also browning inhibitors. In this sense, although it seems that exercise reduces circulating myostatin levels, additional studies combining the effect of exercise on this molecule and on BAT activity in humans should be taken into account, so we can establish evidence of endocrine communication between both tissues, skeletal muscle and BAT.

Follistatin

Follistatin can be secreted by muscle, liver, and other tissues including WAT and BAT[241]. Follistatin binds several members of the TGF-β superfamily including activins and myostatin to neutralize their biological activities[241]. Therefore, the follistatin-mediated suppression of the myostatin signaling has been identified as an important pathway involved in muscle metabolism, differentiation and growth[242]. Besides the inhibition of myostatin action, follistatin likely promotes muscle growth and BAT development by direct activation of Myf5 expression and muscle precursor cells[243]. Moreover, follistatin treatment in muscle leads to increase FNDC5 expression and irisin secretion in mice[244,245]. Indeed, several studies have reported a WAT browning effect of follistatin in murine models[241,246].

Exercise increases follistatin levels in humans, although the effect may be dependent on the type and intensity of exercise[206,207,242,247] (**Table 13**). For instance, Perakakis et al (2018) found that two different exercise intensities (i.e. 70% and 90% of VO_2max) acutely increase follistatin levels, independently of the presence of the metabolic syndrome[242]. Moreover, Sargeant et al (2018) showed that circulating follistatin levels increased after a moderate-intensity bout of exercise (i.e. 60 minutes at 60% VO_2max) and remained elevated for at least 6 hours[206]. Novel approaches combining different exercise type and intensities, follistatin circulating levels and

BAT activity could be considered to study the possible role of follistatin as an exerkine and also, to confirm if follistatin is capable to inhibit myostatin in human adults *in vivo* through exercise. Other studies should be also relevant to investigate the source of circulating follistatin in response of exercise.

Follistatin-like protein 1

Follistatin-like protein 1 is a glycoprotein of the follistatin family proteins group[248]. Fstl-1 is secreted by skeletal muscle to promote endothelial cell function through activation of Akt-eNOS signalling in mice[249] and humans[250]. Moreover, recent studies suggest that follistatin-like protein 1 stimulates BAT thermogenesis through β3-adrenergic activation in mice[251], and that was positively correlated with levels of UCP1 and β3-adrenergic receptors expression[251].

Exercise seems to increase follistatin-like protein 1 circulating levels (**Table 13**). Gorgens et al (2013) observed a 22% increase in follistatin-like protein 1 serum levels immediately and 30 minutes after a 60-minute cycling bout in trained healthy men[250]. Similar results were obtained by Xu et al (2020)[252]. Levels of follistatin-like protein 1 also increased after an acute sprint interval exercise in healthy young men[253]. It seems that follistatin-like protein 1 response to a single bout of exercise displays an acute response, as this protein levels returned to baseline levels after exercise. Although there are currently few studies involving follistatin-like protein 1 as a possible exerkine, the findings appear to be robust in terms of the effect of exercise on circulating levels of the molecule. Therefore, it would be interesting to develop further studies as well as to include BAT activity in the study to clarify whether it may indeed have an endocrine function on this tissue, thus showing a BAT-skeletal muscle communication.

Brain derived neurotrophic factor (BDNF)

Brain derived neurotrophic factor (BDNF) is a neurotrophin mainly expressed in the hippocampus that stimulates synaptic plasticity and memory in humans[254] and plays also a role in energy homeostasis[255]. In mice, BDNF is secreted in response to an enriched environment (i.e. the presence of mazes and toys) and exercise, resulting in WAT browning in both cases[215]. Importantly, the artificial inhibition of BDNF during exercise inhibited the exercise-induced WAT browning[31]. The BDNF effects seems to be partially mediated by the expression of PGC-1α and FNDC5[256]. Several studies have shown an increase in BDNF circulating levels after both moderate and high-intensity aerobic exercise across different populations[257–260], whereas the acute effect of resistance training remains unclear and controversial[261–265] (**Table 13**). Therefore, additional studies are needed to clarify the effect of chronic and resistance exercise on BDNF

levels, as well as the influence of this protein on BAT function in humans, since it seems to promote WAT browning in mice.

Adiponectin

Adiponectin is an hormone secreted by WAT with anti-inflammatory and cardioprotective roles[266] and is likely to stimulate WAT browning through the recruitment of M2 macrophages *in vitro*[267]. In humans, adiponectin circulating levels seem to be positively associated with cold-induced BAT glucose uptake[268].

The effect of exercise on adiponectin circulating levels is controversial. Some studies report that adiponectin plasma levels remains unchanged after exercise[187,269–271], whereas other suggest an increase only in trained subjects[272–274]. However, chronic endurance exercise could improve adiponectin levels in obese young females[275] (**Table 13**). Further studies are required to confirm the role of exercise on circulating levels of adiponectin, not only in trained subjects, but also in sedentary people, who represent a large percentage of society who are more prone to suffer from metabolic diseases. It would also be useful to study how increased circulating levels of adiponectin affect WAT and BAT signaling pathways, and thus WAT browning and BAT activation, respectively.

Leptin

Leptin is mainly produced and secreted by WAT[276]. Indeed, leptin serum concentrations are tightly correlated with fat mass[277]. Leptin regulates energy homeostasis, both by suppressing appetite and stimulating energy expenditure, binding to its receptor in the hypothalamus[276]. Leptin seems to activate BAT through increasing sympathetic tone[278–280], whereas leptin deficiency results in impaired BAT function[281,282]. Leptin administration increases FNDC5 expression in skeletal muscle, but paradoxically, decreases FNDC5 expression in WAT and WAT browning[283].

The leptin response to exercise seems to be consistent across the data reported in the literature, but there is still some discussion (**Table 13**). Both moderate[284,285] and high-intensity[273,284] exercise seems to evoke no change or a little decrease[268,274,285–289] in leptin levels (**Table 13**). While it seems robust that exercise has a negative effect on circulating leptin levels, the impact of this on BAT activation or WAT browning in humans should be further investigated.

Vascular Endothelial Growth Factor A

Vascular Endothelial Growth Factor (VEGF) is a growth factor family that stimulates angiogenesis and vasculogenesis, inducing vascular endothelial cell activation, proliferation and migration[290]. Importantly, one of the members of this protein family, VEGF-A, is secreted by BAT and may act in a paracrine way to regulate vascularization and activate thermogenesis[77,78,291]. Several studies have reported an increase in circulating VEGF-A after a bout of aerobic and resistance exercise in both men and women[291–293], but some of them did not see any effect[294–296] (**Table 13**). Although the available evidence is still preliminary, it might be that the exercise-induced secretion of VEGF-A is also a factor contributing to BAT activation and/or WAT browning. Thus, additional research is required.

Angiopoietin-like 4

Angiopoietin-like protein 4 (ANGPTL4) belongs to a family of multifunctional glycoproteins that inhibit lipoprotein lipase[297]. This protein is mainly secreted by WAT to facilitate the uptake of triglycerides-derived fatty acids by tissues with higher energy demand, such as skeletal muscle and BAT[298–300]. In addition to the nutritional status, the production of ANGPTL-4 is regulated by exercise in humans[301–303] (**Table 13**). A recent study showed that acute endurance exercise increased circulating ANGPTL4 levels in healthy males, being the liver the main secreting site[302]. In another study, an increase in circulating ANGPTL-4 was observed after a 100km ultra-marathon running in healthy men[304]. Whilst it seems plausible that acute exercise of moderate intensity increases circulating levels of ANGPTL4 in humans, it is unknown whether chronic exercise of higher intensity and endurance is able to modulate circulating levels of the protein as well. Furthermore, the effect of ANGPTL4 on human BAT should be further investigated.

METABOLITES

β-aminoisobutyric acid

β-aminoisobutyric acid (BAIBA) is a non-protein amino acid derived from valine catabolism, a very active process in skeletal muscle[305]. BAIBA is secreted by skeletal muscle cells in response to PGC-1α activity[306]. Roberts et al (2014) showed that BAIBA increases the expression of thermogenic genes in WAT, facilitating the browning process[306]. These effects were similar in human-induced pluripotent stem cells and in white adipocytes derived from human pluripotent cell lines[306].

The effects of exercise on BAIBA are controversial. It has been reported an increase in BAIBA levels after 20 weeks of highly controlled endurance exercise training[306]. Similarly, another study showed that 1 hour of low intensity aerobic exercise increases plasma levels of

BAIBA in recreatinally active humans [307]. In contrast, a bout of endurance exercise of moderate intensity failed to induce a significant effect on serum BAIBA in untrained adults[308], and BAIBA levels were not changed after 6 weeks of aerobic exercise training in American Indian children[309] (**Table 13**). Further studies should be conducted to elucidate the role of exercise on circulating BAIBA levels, as well as the type of stimulus and population characteristics. Also, BAT variables should be taken into account to clarify the role of BAIBA in human adults.

Lactate

Lactate is a product of anaerobic glycolysis and is secreted by muscle during high-intensity exercise[310] (**Table 13**). Lactate seems to be involved in BAT metabolism since murine brown adipocytes overexpress the monocarboxylate transporter 1 in response to exercise, promoting the lactate internalization into brown adipocytes[311]. As a consequence, lactate-induced browning of WAT is thought to be mediated by a change in intracellular redox state (NADH-to-NAD+ ratio)[312]. However, lactate might induce WAT browning through the FGF21 expression in brown adipocytes, which likely acts in an autocrine manner to induce browning[313]. The lactate response to exercise is well recognized in sport physiology, being aerobic and resistance exercise able to elicit a significant and rapid increase in an intensity-dependent manner[314,315] (**Table 13**). While the effect of exercise on lactate levels are well known, the role of this increase on human BAT activity and WAT browning is unknown.

β-hydroxybutyrate

β-hydroxybutyrate is a ketone body[316], which seems to promote WAT browning through a change in intracellular redox state[312]. Interestingly, dietary β-hydroxybutyrate promotes WAT browning in animal models[317]. It has also been pointed that ketogenic diets upregulate BAT UCP1 expression[318,319]. In contrast, a recent study showed that β-hydroxybutyrate does not promote adipocyte browning in isolated visceral and subcutaneous fat cells[320]. β-hydroxybutyrate is used as fuel source when glucose availability is reduced[316]. During exercise, β-hydroxybutyrate circulating concentrations are commonly decreased, as a consequence of a higher muscle uptake than hepatic production[316]. Nonetheless, it is quite common to observe increased circulating levels of β-hydroxybutyrate during prolonged exercise or after intense exercise[316,321,322] (**Table 13**). It is of note that the ketone bodies response to exercise seems to be dependent on the level of training and diet[323]. Therefore, although the available evidence is still preliminary, it would be valuable to consider this metabolite in future studies to determine the effect of different types, duration and intensity of exercise and its effect on BAT activity in human adults.

12,13-dihydroxy-9Z-octadecenoic acid (12,13-diHOME)

The lipokine 12,13-diHOME promotes an increase in fatty acid uptake, lipolysis and thermogenesis in BAT[159]. 12-13-diHOME, as well as the enzymes involved in its synthesis, seems to be released from BAT after 1h of cold exposure in rodents and humans[159]. Stanford et al (2018) reported an increase in 12,13-diHOME levels immediately after exercise which returned to baseline 1 hour after exercise in young men and women[96] (**Table 13**). They also observed higher 12-13-diHOME expression in active subjects comparing to sedentary ones, independently of BMI[96]. Moreover, they elegantly proved that BAT was the 12-13-diHOME secreting site during exercise by observing the absence of the exercise-induced increased in mice whose BAT had been surgically removed. Another study in trained cyclist who performed a 75 km moderate-intensity test showed that 12,13-diHOME levels increase just after the exercise, persisting elevated at least during 90 minutes[324]. These findings suggest that 12-13-diHOME may be secreted by BAT during exercise impacting skeletal muscle metabolism. However, Jurado-Fasoli et al (2022) reported that both acute (endurance and resistance exercise) and chronic exercise (a 24-week supervised exercise intervention) did not enhance the plasma levels of 12,13-diHOME in sedentary young adults[325]. The differences observed between studies may be due to the training status and capacity of the individuals. Since the controversial results observed during last years, further research is needed to test different types of exercise and different populations. If 12,13-diHOME is confirmed to be an exerkine, it would impact on knowledge of the crosstalk between BAT and skeletal muscle.

As discussed in this section, several tissues secrete molecules, termed exerkines, in response to exercise, which may target distant organs. Some of these exerkines might exert their function by activating BAT or by stimulating WAT browning. However, almost all available scientific evidence is only in rodents and *in vitro* models, with very few findings in humans. Therefore, further research in humans is urgently needed to clarify, on the one hand, the type of exercise, duration and intensity that modulate the circulating levels of these exerkines, and on the other hand, the impact they have on BAT and WAT browning. Given the complexity of the latter objective, it would be quite useful and interesting to study the association between circulating levels of these exerkines and variables related to BAT volume and activity in adult humans. This will clarify whether exercise actually exerts a positive effect on BAT and WAT browning and, more crucially, will identify those molecules which can be mimicked by pharmacological drugs having a beneficial metabolic impact in different patients. Therefore, this field of study aims to offer a new strategy for both the prevention and treatment of metabolic diseases.

Because of this necessity in BAT research, the Present Doctoral Thesis aims to identify 16 exerkines released into the bloodstream in sedentary young adults. This study is addressed in

Study II of Chapter 2.2, where these 16 exerkines are investigated after one session of acute endurance aerobic exercise, another session of acute resistance exercise, and after a 24-week intervention program. In addition, we also examined whether these exercise-induced changes are associated with BAT volume and activity in these participants. In this way, the aim is to establish a relationship between the modulation of the circulating levels of these exerkines and BAT function in humans.

Table 13. *Exercise effect on circulating levels of endocrine molecules that are able to regulate brown adipose tissue metabolism and/or white adipose tissue browning, or communicate BAT with other tissues during exercise, in humans.*

	Moderate-intensity aerobic exercise		High-intensity aerobic exercise		Resistance exercise		Participants
	Exercise	Recovery	Exercise	Recovery	Exercise	Recovery	
Protein hormones							
Norepinephrine	↑ [326–330]	↷ [326–330]	↑ [190,331–335]	↷ [190,331–335]	↑ [335–339]	↷ [335–339]	Lean and obese children [338] Lean young men [328,330–332,334,335] and women [328] Lean [190,338], overweight [327,333] and obese [326,327] middle-aged men T1DM lean and overweight middle-aged adults [336,339] Healthy lean elderly adults [337]
ANP	↑ [190,327,330,340,341]	↷ [190,327,330,340,341]	↑ [188,190,342,343]	↷ [188,190,342,343]	~ [341]	~ [341]	Healthy lean elderly and young adults [342]

Irisin	~ [344] ↑ [115,345,346]	↷ [115,346]	↑ [194,346–348] ↓ [234]	↷ [194,346]	~ [349,350] ↑ [351]	↓ [349] ↷ [351]	Athletes and lean sedentary young adults [343] Lean [198–200] and obese young men [340] Overweight healthy middle-aged men [188,190,327] and women [327] Obese healthy middle-aged adults [188] Lean young males [194,234,344–346,348–351] and women [348] Pregnant women [347] Lean and overweight sedentary middle-aged men [115]
FGF21	~ [205–207,247,344,352–355]	↑ [205–207,247,344,352–355]	~ [207,355] ↑ [208]	↑ [207,355]	~ [352,356]	~ [352,356]	Lean [205,207,247,344,352–356] and obese [205] young men Lean and overweight middle-aged men [206] Lean elderly men [354]

						Male runners [208]
IL-6	↑ [357–363]	↑ [359–361]	↑ [348,360,362,363]	↑ [360]	↑ [356,362,364]	↑ [356,364–367]	Lean young men [348,356,357,360,365,366] Lean young adolescents [358] Overweight middle-aged adults [359,363] T1DM lean and overweight middle-aged adults [367] Lean and obese T2DM middle-aged men [361,362] Obese elderly women [364]
Meteorin-like	↑ [211]	?	?	?	?	?	Healthy active overweight young women[211]
Musclin	?	?	?	?	?	?	
GDF15	↑ [223,225]	↑ [223,225]	↑ [208,223,224,226]	↑ [208,223,224,226]	↑ [223]	↑ [223]	Healthy active young men [225]

						Healthy trained males and elite male players [208,223,224]
Myostatin	↑ [344]	↓ [344]	~ [234]	↓ [234]	~ [368]	↓ [235] ~ [368]	Lean young men [234,235,344] Lean middle-aged men [368]
Follistatin	~ [206,207,247,344]	↑ [206,207,247,344]	~ [207]	↑ [207]	~ [349,368]	↑ [349,368]	Lean young men [207,247,344,349] Lean middle-aged men [206,368] Overweight middle-aged men [206]
Follistatin-like 1	↑ [250,252]	↷ [250]	↑ [253]	↷ [253]	?	?	Healthy lean trained adult men [250] Healthy lean young men [252,253]
BDNF	↑ [257,347,369–380] ~ [381,382]	↷ [369,372,373,375–377] ~ [381]	↑ [369,374,378,381,382]	↷ [369,381] ~ [263,264]	~ [261,262] ↑ [265]	?	Metabolic syndrome and healthy adults [373] Healthy young male athletes [257,370,374,378,381,382] Healthy young adults [262–265,372,376,377]

						Pregnant and post-partum women [347] Elderly sedentary lean/overweight women [379] Panic-disorder adults [380] Healthy trained/untrained adults [261] Major depressed young adults [369] Young healthy sedentary men [371]
Adiponectin	~ [187,269–272]	~ [187,269,271] ↑ [272]	~ [269,273,274]	↑ [273,274] ~ [269]	?	?	Healthy lean young men [111,115] Healthy moderate active adults [270] Healthy young lean active men [187,272–274] Overweight young men [271]

Leptin	~ [269,270,284,285,383,384] ↓ [286,287,385]	↓ [284,383,385] ↷ [287]	↓ [286] ~ [273,274,285]	↓ [274,285] ~ [273]	~ [386]	↓ [386]	Healthy moderately active adults [270] Healthy young trained lean men [272–274,284,286,385] Healthy young lean men [285,386] Healthy trained men [383] Premenopausal obese adult women [384] Healthy sedentary adults [287]
VEGFA	↑ [292] ~ [294,295,387]	~ [294–296] ↑ [292,387]	↑ [293,388]	↷ [293]	↑ [389] ~ [390]	↑ [389,390] ~ [296]	Healthy young men [292,294,296] Healthy young trained men[295,387]. Healthy young trained women[293] Healthy lean middle-aged adults[388]. Healthy young women[389]. Healthy young sedentary men [390].

ANGPTL4	~ [391] ↑ [301,302,304]	↑ [302,391]	?	?	?	?	Healthy lean and obese sedentary men [391] Healthy lean and overweight adult men [301] Healthy young men [302] Ultramarathon male runners [304]
Metabolites							
BAIBA	↑ [307] ~ [308]	↑ [307] ~ [308]	?	?	?	?	Healthy young active adults [307] Healthy young untrained men [308,392]
β-hydroxybutyrate	~ [316,393,394] ↑ [392,393,395] ↓ [392]	↑ [393,395,396] ~ [393]	~ [397]	↑ [397]	?	?	Healthy lean young trained/untrained men [393] Healthy young trained/untrained men [316,395,397] Obese middle-aged men [394]

Lactate	↑ [314,315]	↷ [314,315]	↑ [314,315]	↷ [314,315]	↑ [315]	↷ [315]	Consistent response among different populations
12-13-diHOME	↑ [96,324] ~ [325]	↷ [96,324]	? ~ [325]	?	?	?	Young/elderly sedentary/active adults [96] Young healthy male cyclists [324] Sedentary young adult [325]

Symbols: (↑) Increase, (↓) decreased, (~) unchanged, (?) unknown, (↷) return to basal levels. Different symbols are used within the same cell when controversial results have been published.

Abbreviations: ANGPTL4: Angiopoietin-like 4; Baiba: Beta aminobutyric acid; BDNF: Brain derived neurotrophic factor; β-OH-butyrate; GDF15: Growth differentiation factor 15; FGF21: Fibroblast growth factor 21; Fstl-1: Follistatin protein like 1; Mtrn-like: Meteorin like; T1DM: type 1 diabetes mellitus; T2DM: type 2 diabetes mellitus; VEGF: Vascular endothelial growth factor A; 12,13-diHOME: 12,13-dihydroxy-9Z-octadecenoic acid.

2.2 Exercise-induced changes on exerkines that might influence brown adipose tissue metabolism in young sedentary adults

BACKGROUND

Following preclinical evidence, it could be hypothesized that exercise also induces WAT browning and/or regulate BAT metabolism in humans. Unfortunately, the available evidence in humans is insufficient and controversial, mainly due to the lack of longitudinal studies with adequate statistical power- One of the issues that remains to be addressed is characterizing the acute and chronic effect of different types of exercise (i.e., endurance and resistance exercise) on the circulating levels of exerkines that are known to induce WAT browning and/or regulate BAT metabolism in humans. It would also be noteworthy to stablish the relation between the exercise-induced changes in circulating levels of exerkines and BAT volume and activity.

This study aims to analyse the acute (endurance and resistance) and chronic (combined endurance and resistance) effect of exercise on the circulating concentrations of 16 exerkines that have been shown to regulate BAT metabolism and/or WAT browning *in vitro* or in preclinical models (adiponectin, leptin, ANP, pro-ANP, BAIBA, lactate, norepinephrine, BDNF, IL6, meteorin-like, follistatin, FSTL1, irisin, myostatin, musclin, and FGF21), in a cohort of young sedentary adults. We also explored the associations between the exercise-induced changes in the concentration of these exerkines and BAT volume, glucose uptake and mean radiodensity, assessed after an individualized cold exposure. Moreover, we explored whether the exercise-induced changes in the circulating exerkines are related to body composition, sex, circulating cardiometabolic risk factors, or physical fitness.

MATERIALS AND METHODS

Study design

This work has been conducted under the framework of the ACTIBATE study [119], a randomized controlled trial designed to study the effect of an exercise program on BAT volume and activity (ClinicalTrials.gov ID: NCT02365129). Inclusion criteria were 18-25 years old, reporting no more than 20 minutes of moderate-vigorous physical activity in a maximum of 3 days per week, absence of cardiometabolic disease, non-smoker, not taking any medication affecting energy metabolism, and having stable body weight during the previous three months. This study was conducted following the last version of Declaration of Helsinki. The protocol and written

informed consent were approved by the Ethics Committee on Human Research of the University of Granada (nº 924) and "Servicio Andaluz de Salud".

First, we analysed the acute effect of endurance and resistance exercise on the circulating levels of 16 exerkines in 10 participants (4 men, 6 women) before and 3, 30, 60 and 120 minutes after a single exercise bout, onwards referred as *discovery study*. Those exerkines that were changed by acute endurance exercise were further studied in another 28 participants only before exercise and at the time point when the molecule was altered in the *discovery study*. These individuals, together with the 10 participants of the *discovery study* resulted in a total of 38 participants (10 men, 28 women), onwards referred as *confirmatory study.* In this group, we also analysed the association between the exercise-induced change in the selected molecules and BAT-related variables, body composition, cardiometabolic risk factors, and physical fitness. Finally, we tested the effect of a 24-week exercise training program, including endurance and resistance exercise, on the circulating levels of 16 exerkines using samples collected before and after the exercise program in 110 participants (35 men, 75 women) during resting and fasting conditions, onwards referred as *chronic study*. The study design is summarized in **Figure 13**.

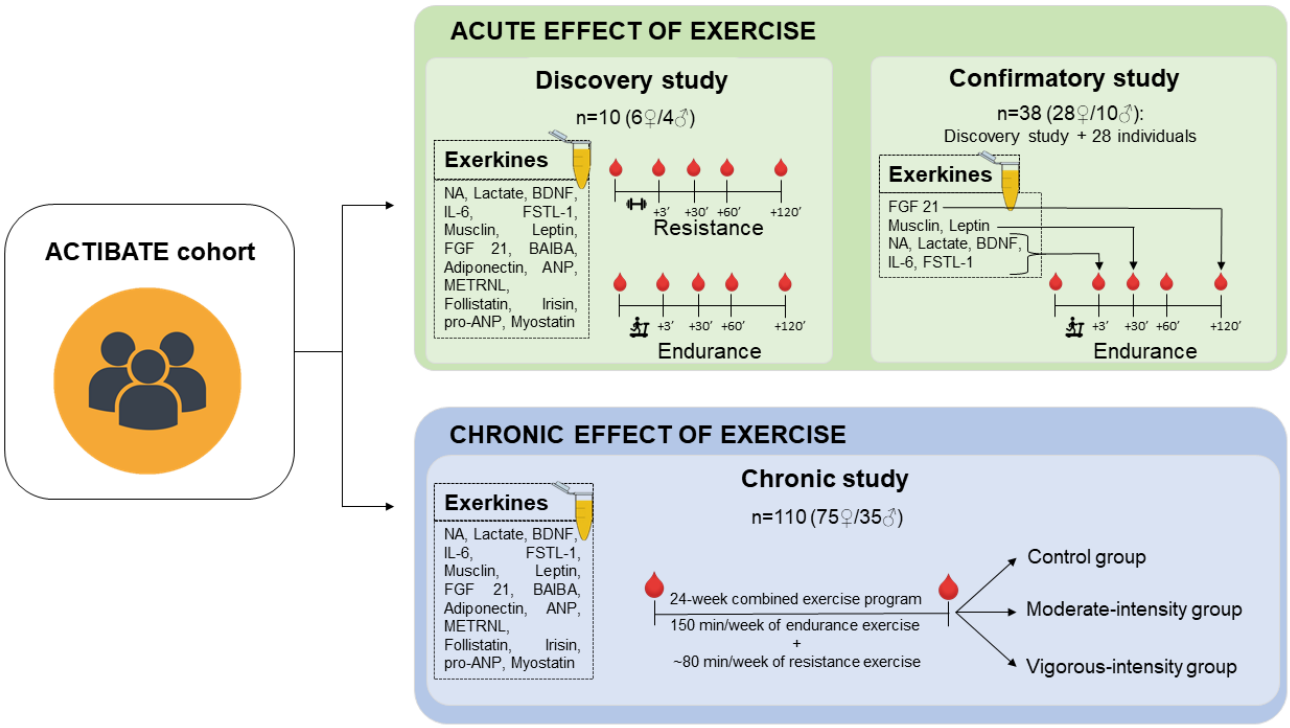


***Figure 13**. Study design. We analysed the acute effect of endurance and resistance exercise on the circulating levels of 16 exerkines using plasma samples collected from 10 participants before and 3, 30, 60 and 120 minutes after a single exercise bout, conducted during the baseline assessment of the ACTIBATE study (discovery study). Those exerkines that were changed by acute endurance exercise were further determined in another 28 participants only before exercise and at the time point when the molecule was altered (confirmatory study). We also tested the chronic effect of exercise on plasma samples collected 1-3 weeks and 4-5 days after a 24-week exercise training program*

Descriptive characteristics of the participants are presented in **Table 14**. The training program was performed in four different waves (both in 2015 and 2016): i) from September to April, ii) from October to April, iii) from October to May, and iv) from November to May.

Table 14. *Characteristics of the study participants.*

	Acute effect Discovery study (n=10)		Acute effect Confirmatory study (n=38)		Chronic effect Chronic study (n=110)	
	Mean	SD	Mean	SD	Mean	SD
Demographics						
Sex (Male/Female)	4/6		10/28		35/75	
Age (years)	21.5	2.3	22.1	2.4	22.1	2.2
Body composition						
Weight (kg)	68.0	14.1	66.2	12.4	70.2	15.3
Height (cm)	168.9	8.9	166.4	8.0	167.9	8.5
Body mass index (kg/m^2)	23.7	3.5	23.9	3.9	24.7	4.2
Waist circumference (cm)	76.1	9.1	77.5	11.2	81.4	12.9
Lean mass (kg)	41.1	8.1	39.6	7.8	41.5	9.3
Fat mass (kg)	23.3	6.8	23.1	8.7	24.9	8.5
Fat mass (%)	34.7	5.2	35.0	9.0	34.8	7.5
VAT mass (g)	294	96	311	176	346	176
Cardiometabolic risk factors						
Glucose (mg/dL)	85.6	6.9	87.2	5.8	87.2	6.3
Insulin (µUl/mL)	6.1	2.7	7.4	4.1	8.2	4.1
HOMA-IR	1.3	0.6	1.6	1.0	1.8	1.0
Triacylglycerol (mg/dL)	68.2	17.4	89.0	70.0	83.1	47.3
Cholesterol (mg/dL)	168.7	24.3	171.1	41.8	162.7	30.6
HDL cholesterol (mg/dL)	53.6	8.1	53.3	9.5	52.5	11.9
LDL cholesterol (mg/dL)	101.5	22.1	102.1	32.9	94.3	25.7
Systolic BP (mmHg)	114.7	11.2	114.8	10.5	117.5	11.6
Diastolic BP (mmHg)	71.2	9.9	70.7	7.7	71.4	7.3
Physical fitness						
Time to exhaustion (min)	16.3	3.5	14.6	3.94	15.3	3.4
VO_2peak (mL/kg/min)	42.4	7.2	41.9	8.0	40.9	8.3
Hand grip strength (kg)	35.1	9.9	31.2	7.6	31.1	7.5
RM leg press (kg)	222.8	50.5	204.3	50.2	197.8	64.1
RM bench press (kg)	35.9	11.5	30.4	10.5	31.1	14.4
Brown adipose tissue						
BAT volume (mL)	70.5	62.8	71.8	45.8	69.1	55.8
BAT SUVmean	3.5	2.4	4.0	1.9	3.8	1.9
BAT SUVpeak	11.3	8.7	12.6	9.0	11.1	8.2
BAT mean radiodensity (HU)	-59.4	12.0	-58.5	11.8	-59.9	11.4

Data presented as mean and standard deviation (SD), except for sex. Abbreviations: VAT: visceral adipose tissue; HOMA-IR: homeostasis model assessment of insulin resistance; HDL: high-density lipoproteins; LDL: low-density lipoproteins; BP: blood pressure; VO_2peak: Maximal oxygen uptake; RM: 1 Repetition Maximum; BAT: Brown adipose tissue; SUV: Standardized uptake value; HU: Hounsfield units.

Acute exercise sessions

Plasma samples for the *discovery and confirmatory studies* were collected during 2 exercise sessions, one involving endurance exercise and the other resistance exercise, taking place during the baseline assessment period of the ACTIBATE study. Both sessions were carried out in 3-5h fasted state after avoiding stimulants as well as moderate (24h before) and vigorous (48h before) exercise [119].

The endurance exercise session consisted of a maximum effort test on a treadmill (Pulsar treadmill, H/P/Cosmos Sports & Medical GmbH, Nussdorf-Traunstein, Germany) as previously described [112]. For warming up, participants walked at 3 km/h for 1 minute and at 4 km/h for 2 minutes (0% grade). Subsequently, the test started by walking at 5.3 km/h and 0% grade. From that moment on, the treadmill grade was increased by 1% every minute, until volitional exhaustion was reached. Immediately after, participants started a 5-minute recovery. Participants 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).

The resistance exercise session consisted of a maximum isometric strength test in leg press, a handgrip strength test, and two one repetition maximum (1-RM) tests in bench and leg press, as described elsewhere [112]. Participants first completed the maximum isometric strength test in leg press, performing two 3-second repetitions, two minutes apart. Later, participants performed the handgrip strength test by completing two repetitions with each hand, one minute apart. Then, participants completed the RM test in the leg press machine and the bench press (Power rack, Model 3111, Keiser Corporation, Fresno CA, USA). After warming up, they were instructed to perform one set of 8 repetitions selecting the resistance with which they could perform 15 repetitions as much. After a 1-minute recovery, the resistance load was increased by the study personnel, aiming to set a load with which the participant could perform <10 repetitions and they did as many repetitions as possible. The maximum number of attempts was three. A more detailed description can be found elsewhere [112].

Before each exercise session, an intravenous catheter was inserted in the antecubital vein. Blood was collected in 2 tubes of 4 ml, one containing ethylenediamine tetra-acetic (EDTA) and the other one heparin, immediately before, and 3, 30, 60, and 120 minutes after the end of the exercise bout. Blood was immediately centrifuged (10 minutes, 3000 rpm, 4ºC) and plasma aliquots stored at -80ºC until analyses.

Combined exercise training program

The exercise training program of the ACTIBATE study has been described in detail elsewhere [119]. Shortly, it consisted of a 24-week exercise program with 150 min/week of endurance exercise and ~80 min/week of resistance exercise. Participants (35M/75F) were randomized into three groups (control, moderate-intensity and vigorous-intensity). The control group did not perform any exercise. For the endurance exercise, the vigorous-intensity group performed 75 min/week at 60% of heart rate reserve (HRres) and 75 min/week at 80% HRres, whereas the moderate-intensity group performed all endurance training at 60% HRres. Concerning strength training, both groups completed 2 sessions/week consisting of exercises localized in the major muscle groups. Participants of the moderate-intensity group trained at 50% of 1-RM while the vigorous-intensity group at 70% 1-RM. Blood samples were collected in Vacutainer Tubes® 1-3 weeks before and 4-5 days after the training program, after an overnight fast and avoiding physical exercise during the previous 48h for vigorous-intensity and 24h for moderate-intensity. Blood was immediately centrifuged (10 minutes, 3000 rpm, 4ºC), and serum and plasma aliquots stored at -80ºC until analyses.

Exerkines

All exerkines were determined using plasma samples containing EDTA except for meteorin-like, that was determine in plasma samples containing heparin. BAIBA was determined by high-performance liquid chromatography (HPLC), according to the previously described methodology [398], by Agilent Liquid Chromatography System series 1100 (Agilent Technologies, USA) using columns from Phenomenex Luna HILIC (100x30mm, Phenomenex, CA, USA). The kits used for the determination of the other exerkines plasma levels are pointed in **Table 15**.

Table 15. *Determination of exerkines plasma levels (except BAIBA).*

Exerkine	Method	Kit reference	Intra-assay CV
Meteorin-like	ELISA	DY7867-050 (R&D System, Minneapolis, MN, USA)	5.4
ANP		CEA225HU (Cloud-Clone, Wuhan, China)	11.3
Pro-ANP		CSBEQ028056HU (Cusabio Biotech, Wuhan, China)	15.4
FGF21		RD191108200R (Biovendor, Brno, Czech Republic)	10.1

Norepinephrine		BA E-6200 (LDN, Nordhorn, Germany)	17.8
BDNF	XMap technology (Luminex Corporation, Austin, TX)	HMYOMAG-56K (Milliplex Map Kit; Millipore, Billerica, MA)	5.4
Myostatin			10.1
Musclin			8.1
Irisin			17.3
FSTL1			7.7
Adiponectin		HADK1MAG-61K (Milliplex Map Kit; Millipore, Billerica, MA)	9.0
Leptin			8.3
Follistatin		HAGP1MAG-12K (Milliplex Map Kit; Millipore, Billerica, MA)	5.1
IL-6		HSTCMAG-28SK (Milliplex Map Kit; Millipore, Billerica, MA)	11.5
Lactate	Colorimetric method	1001330, (Spinreact, Girona, Spain).	13.1

Abbreviations: CV: coefficient of variation; ELISA: enzyme-linked immune-absorbent assay; ANP: atrial natriuretic peptide, FGF21: fibroblast growth factor 21; BDNF: brain-derived neurotrophic factor; FSTL1: follistatin-like protein 1; IL-6: interleukin 6.

Body composition

Body weight and height were measured using a SECA scale and stadiometer (model 799, Electronic Column Scale, Hamburg, Germany). Lean mass, fat mass, and visceral adipose tissue (VAT) mass were assessed by dual-energy x-ray absorptiometry (HOLOGIC, Discovery Wi, Marlborough, MA). Waist circumference was measured twice with an elastic-plastic tape, and the mean value was obtained. These measures were obtained in overnight fasting conditions before the 24-week exercise program.

Cardiometabolic risk factors

Serum glucose, total cholesterol, high-density lipoprotein-cholesterol (HDL-C), and triacylglycerols were quantified using an AU5832 automated analyser (Beckman Coulter Inc., Brea CA, USA). Low-density lipoprotein-cholesterol (LDL-C) was estimated as [total cholesterol – HDL-C – (triacylglycerols /5)]. Serum insulin was determined by the Access Ultrasensitive

Insulin Chemiluminescent Immunoassay Kit (Beckman Coulter Inc., Brea, CA, USA) and homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as [insulin(μU/mL) x glucose (mmol/L)/22.5].

Systolic and diastolic blood pressure were measured twice on three different days by an automatic sphygmomanometer Omrom M2 (Omron Healthcare, Kyoto, Japan), and the mean was used for the analyses.

Physical fitness

To determine muscular strength, the 1-RM of the bench press and leg press were assessed using the Wathen equation [399]. We also measured handgrip strength using a Takei 5401 digital Grip-D hand dynamometer (Takei, Tokyo, Japan), by calculating the mean between the best attempt performed with each hand. Maximal oxygen consumption (VO_2peak) was determined as the highest observed VO_2 during the maximum effort test, after removing obvious outliers. The time until exhaustion during the maximum effort test was also calculated.

Brown adipose tissue

BAT volume, ^{18}F-Fluoruodeoxyglucose (^{18}F-FDG) uptake, and mean radiodensity were assessed after a 2h personalized cold exposure by a static ^{18}F-FDG positron emission tomography/computed tomography (PET/CT) scan as previously described [400]. We used the standardized uptake value (SUV) threshold proposed in BARCIST 1.0 recommendations, and a fixed radiodensity range of −190 to −10 Hounsfield units.

Statistical analyses

Descriptive data are presented as mean and standard deviation, unless otherwise stated. Data out of the range of detection of the kits were excluded. A P-value <0.05 was considered statistically significant. Some of the variables did not follow a normal distribution (Shapiro-Wilk test <0.05), thus they were log2 transformed.

In the *discovery study*, the acute effect of exercise on the levels of exerkines measured before and 3, 30, 60 and 120 minutes after exercise was analysed by repeated measures analyses of variance (ANOVA). In the *confirmatory study*, paired student t-tests were employed instead. Finally, in the *chronic study*, the chronic effect of exercise on the levels of exerkines was analysed by 2-factor (*Group x Time*) ANOVAs. We also conducted analyses of covariance (ANCOVA) to compare the exercise training-induced changes (i.e., post-intervention – baseline) adjusted for the baseline level of each exerkine. Moreover, we analysed the *Sex x Time* interaction in the *confirmatory study* and *Sex x Time x Group* interaction effect in the *chronic study.* No significant interactions effects were detected, and thus the analyses were conducted combining men and

women. We also explored the sex effect in the three different studies, without finding any statistical significance. Irisin was only detectable in 4 participants and pro-ANP and myostatin in 1 participant (data not shown), so no further analyses were performed.

We used data from the *confirmatory study* to calculate the acute change (Δ) in circulating concentrations of the exerkines, by subtracting the pre-exercise value to the selected post-exercise value (3 minutes for norepinephrine, lactate, BDNF, IL6 and FSTL1; 30 minutes for musclin and leptin; and 60 minutes for FGF21). We then analysed, by simple linear regression (Model I), the association between the Δ in exerkines concentrations and BAT volume, SUVmean, SUVpeak and mean radiodensity. These associations were also tested in multiple linear regression adjusting for the PET/CT scan date (Model II), BMI (Model III), and the baseline exerkines concentrations (Model IV). Similarly, we explored the association of the exercise-induced Δ in exerkines concentrations with body composition, cardiometabolic risk factors and physical fitness, by using simple linear regressions.

RESULTS

Acute effect of endurance and resistance exercise on the circulating concentrations of exerkines

Endurance exercise increased the circulating concentration of BAIBA (5.3 vs 6.0 ppb, P=0.046), lactate (0.9 vs. 5.5 mmol/L, P<0.001), norepinephrine (1.8 vs. 3.5 ng/mL, P=0.004), BDNF (2.5 vs. 4.8 ng/mL, P=0.008), IL6 (4.5 vs. 6.9 pg/mL, P=0.052), and FSTL1 (13.9 vs. 16.7 ng/ml, P=0.042) immediately after exercise (Figure 10). Musclin (254.3 vs. 319.1 pg/mL, P=0.076) and FGF21 (23.5 vs 72.5 pg/mL, P=0.066) were also upregulated by exercise, reaching a peak 30 and 60 minutes after exercise, respectively (**Figure 14**). In contrast, circulating leptin was reduced after the endurance exercise bout (P=0.025), with the strongest effect appearing 30 minutes after the exercise (6.6 vs 5.9 µg/L) (**Figure 14**). The other studied exerkines were not modified by endurance exercise (**Figure 14**). Those exerkines whose concentrations were modified by endurance exercise were studied in a wider cohort for the *confirmatory study* (n=38) at the time they were more severely modified except for BAIBA, since it was detected only in 60% of the samples. The results observed in the *discovery study* were replicated in the *confirmatory study* (**Figure 15**).

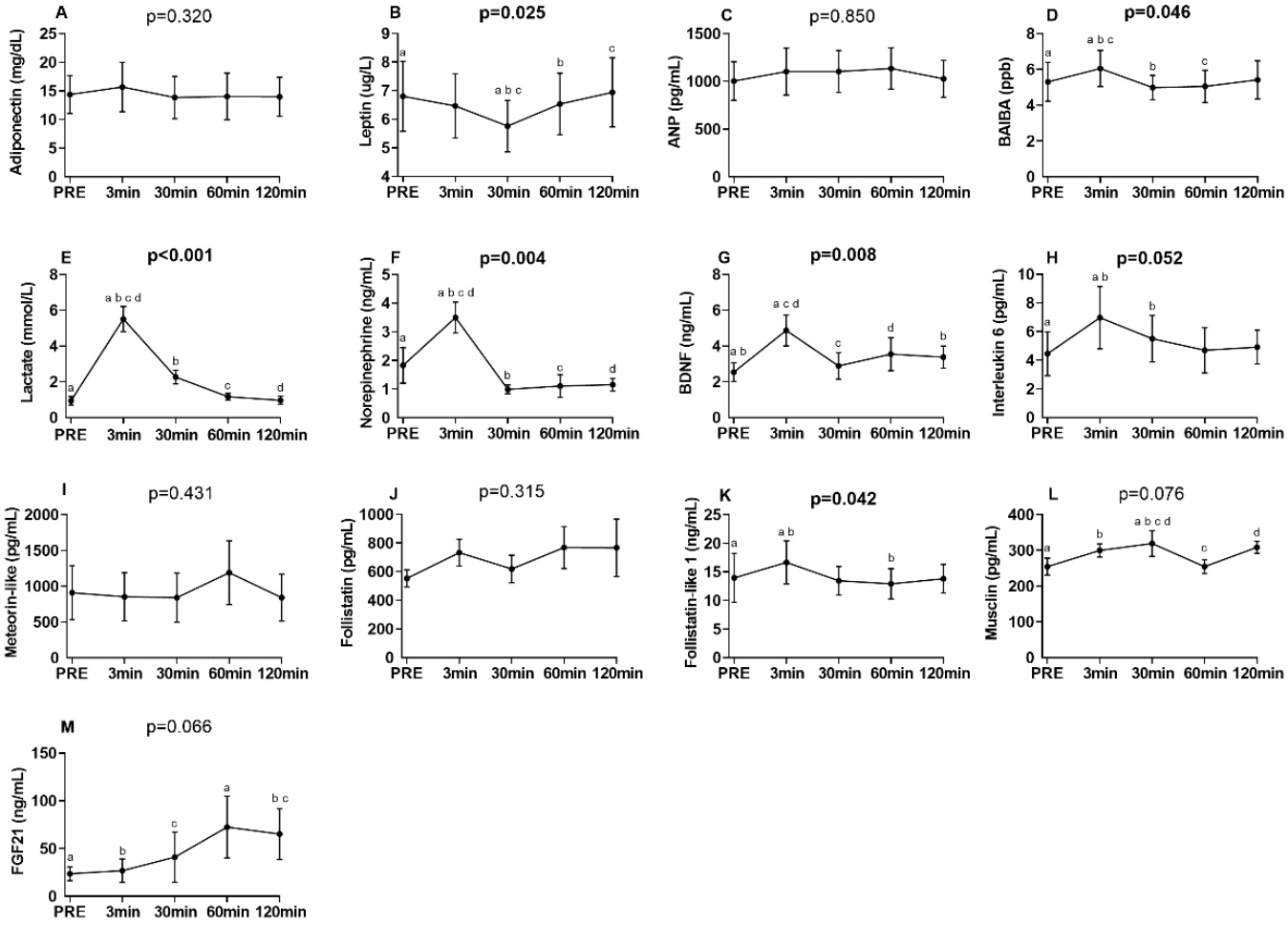


Figure 14. *Acute effect of endurance exercise on circulating concentrations of endocrine signals able to regulate brown adipose tissue metabolism and/or white adipose tissue browning in the discovery study (n=10). Circulating plasma concentrations of adiponectin (A), leptin (B), ANP (C), BAIBA (D), lactate (E), norepinephrine (F), BDNF (G), IL6 (H), meteorin-like (I), follistatin (J), follistatin-like 1 (K), musclin (L), FGF21 (M) were measured before and 3, 30, 60 and 120 minutes after an incremental maximum effort test. P from repeated measured analysis of variance. Error bars indicate standard error. Common letters indicate significant differences in post-hoc analyses. Abbreviations: ANP: atrial natriuretic factor; BAIBA: β-aminoisobutyric acid; BDNF: brain-derived neurotrophic factor; FGF21: fibroblast growth factor 21.*

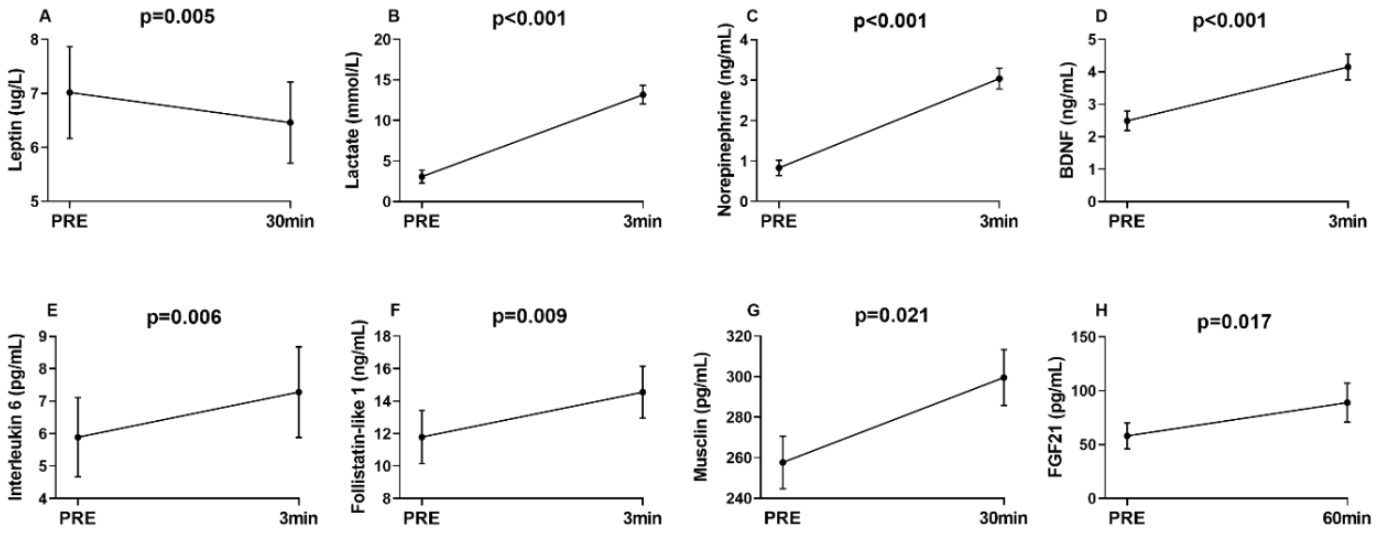


Figure 15. *Acute effect of endurance exercise on circulating concentrations of endocrine signals able to induce white adipose tissue (WAT) browning and/or regulate brown adipose tissue (BAT) metabolism in participants included in the confirmatory study (n=38). Circulating plasma concentrations of lactate (n=38) (B), norepinephrine (n=38) (C), BDNF (n=35) (D), FSTL1 (n=34) (E) and IL6 (n=25) (E) were measured before and 3 minutes after an incremental maximum effort test. Plasma levels of leptin (n=37) (A) and musclin (n=35) (G) were measured before and 30 minutes after an incremental maximum effort test. Circulating plasma concentrations of fibroblast growth factor 21 (n=34) (FGF21) (H) were measured before and 60 minutes after an incremental maximum effort test (n=38). P values from paired student t-test. Error bars indicate standard error. Abbreviations: BDNF: brain-derived neurotrophic factor; FGF21: fibroblast growth factor 21.*

Regarding the acute resistance exercise, we observed an increase of lactate levels (0.8 vs. 3.1 mmol/L, P<0.001) immediately after exercise (**Figure 16**). The rest of circulating exerkines were not modified by resistance exercise (**Figure 16**).

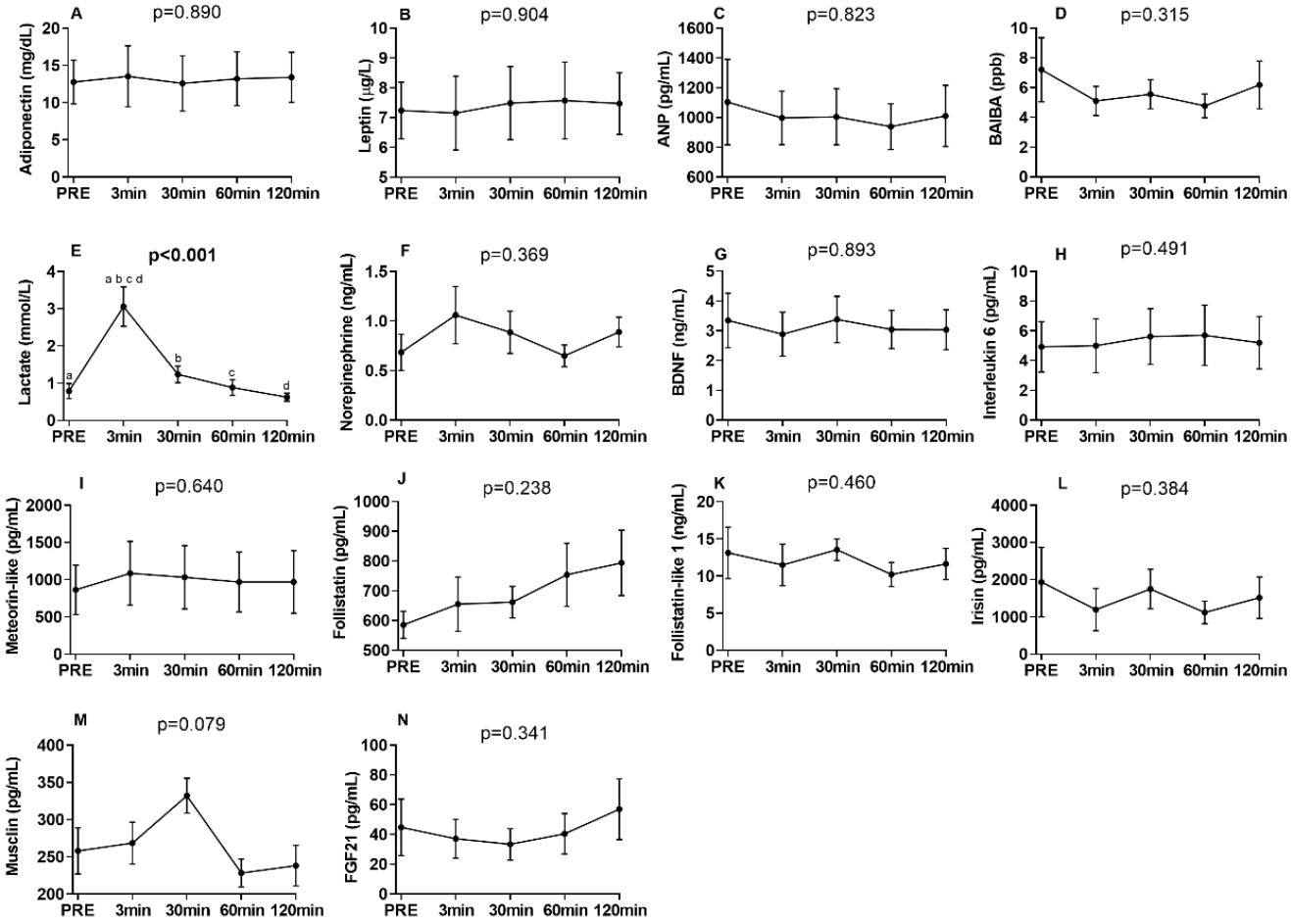


Figure 16. *Acute effect of resistance exercise on circulating concentrations of endocrine signals able to regulate brown adipose tissue metabolism and/or white adipose tissue browning in the discovery study (n=10). Circulating plasma concentrations of adiponectin (A), leptin (B), ANP (C), BAIBA (D), lactate (E), norepinephrine (F), BDNF (G), IL6 (H), meteorin-like (I), follistatin (J), follistatin-like 1 (K), irisin (L), musclin (M) and FGF21 (N) were measured before and 3, 30, 60 and 120 minutes after a session of resistance exercise. P from a repeated measured analysis of variance. Error bars indicate standard error. Common letters indicate significant differences in post-hoc analyses. Abbreviations: ANP: atrial natriuretic factor; BAIBA: β-aminoisobutyric acid; BDNF: brain-derived neurotrophic factor; FGF21: fibroblast growth factor 21.*

Chronic effect of exercise on circulating exerkines

The resting and fasting concentrations of all analysed exerkines were not modified by the 24-week exercise training program (**Figure 17**). Similar results were also obtained when performing analyses of covariance (ANCOVA) (data not shown).

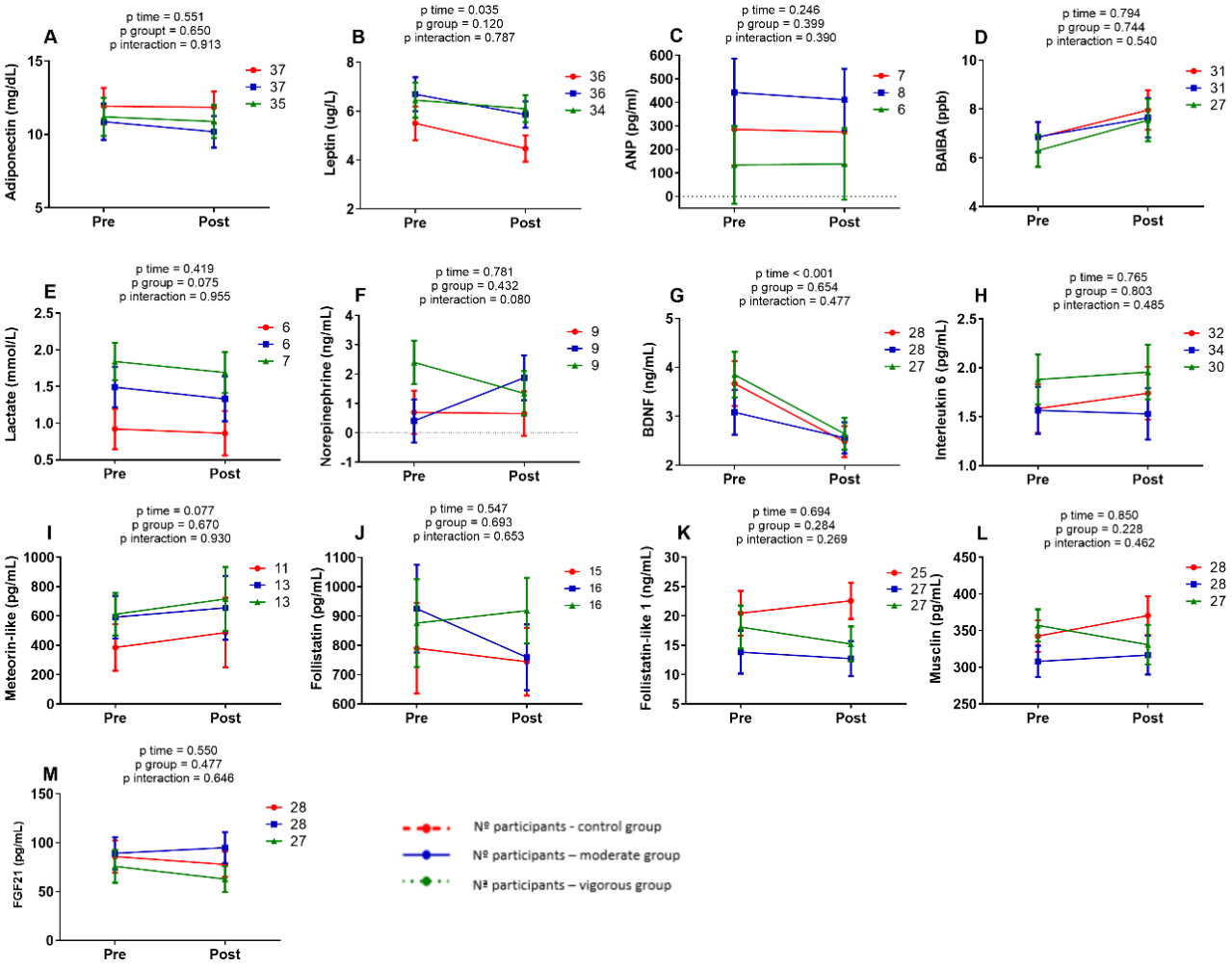


Figure 17*. Chronic effect of a 24-week combined exercise (endurance + resistance) training program on fasting circulating plasma concentrations of endocrine signals able to regulate brown adipose tissue metabolism and/or white adipose tissue browning in the chronic study. Circulating plasma concentrations of adiponectin (A), leptin (B), ANP (C), BAIBA (D), lactate (E), norepinephrine (F), BDNF(G), IL6 (H), meteorin-like (I), follistatin (J), follistatin-like 1 (K), musclin (L) and FGF21 (M) were determined 1-3 weeks before and 4-5 days after the training program. P values from 2-factor (Group x Time) analyses of variance. Circulating levels of pro-ANP, irisin, and myostatin were detected in less than 50% of sample size (data not shown). Error bars indicate standard error. Abbreviations: ANP: atrial natriuretic factor; BAIBA: β-aminoisobutyric acid; BDNF: brain-derived neurotrophic factor; FGF21: fibroblast growth factor 21.*

Association of the endurance exercise-induced changes in exerkines with BAT related variables, body composition, cardiometabolic risk factors and physical fitness

Table 16 shows the association between the endurance exercise-induced acute changes in circulating exerkines and BAT volume, ^{18}F-FDG uptake and mean radiodensity. The endurance exercise-induced change in lactate concentration was positively associated with BAT radiodensity (β=1.050, R^2=0.258, P=0.011) and these results persisted after adjusting for the PET/CT scan

date, BMI, and the baseline lactate concentration (all P≤0.015). When adjusting the analyses for PET/CT scan date, lactate concentration was also positively associated with BAT SUVpeak (β=0.604; R^2=0.538; P=0.033) (**Table 16**). Moreover, when lactate concentration was log2 transformed we also observed associations with BAT volume (β=25.857; R^2=0.196; P=0.011), BAT SUVmean (β=1.017; R^2=0.169; P=0.019), BAT SUVpeak (β=17.395; R^2=0.210; P=0.008), and BAT radiodensity (β=19.808; R^2=0.168; P=0.047) (**Figure 18**). The other studied exerkines were not associated with BAT related variables (**Table 16**).

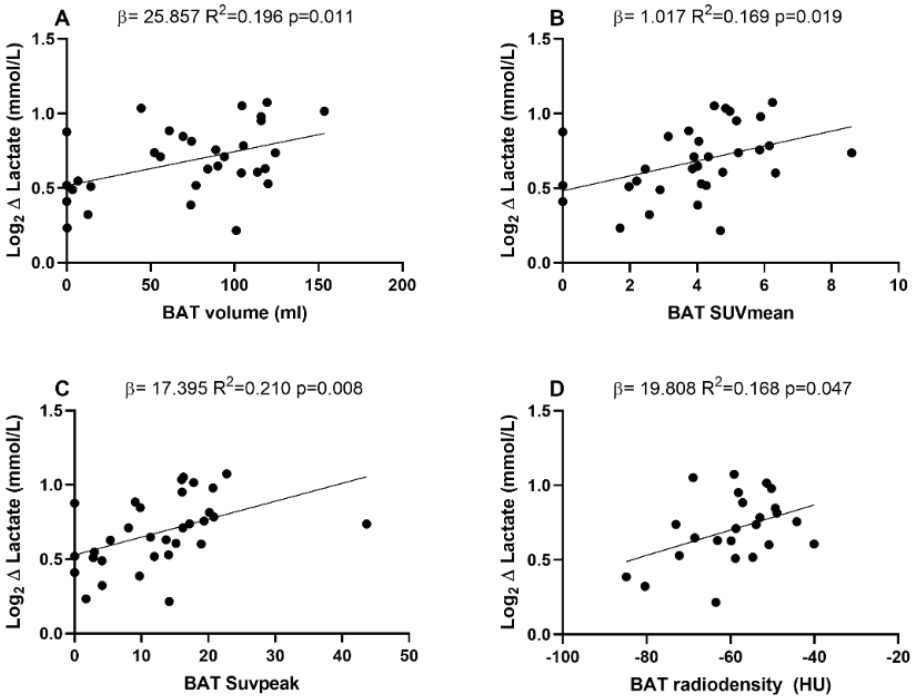


Figure 18. Association between exercise-induced change on lactate concentration (log2 transformed) and BAT volume (A), BAT SUVmean (B), BAT SUVpeak (C), and BAT radiodensity (D). Delta (Δ) of lactate was calculated by subtracting the baseline concentration to the circulating levels obtained 3 minutes after exercise. Unstandardized β, R^2, and P from simple and multiple linear regressions. Abbreviations: BAT: brown adipose tissue, HU: Hounsfield units; SUV: Standardized uptake value.

Table 16. *Associations between endurance exercise-induced changes in the circulating concentration of exerkines and BAT volume, ^{18}F-Fluorodeoxyglucose uptake and mean radiodensity (n=38).*

	Δ Norepinephrine (ng/mL			**Δ Lactate (mmol/L)**			**Δ BDNF (ng/mL)**			**Δ IL6 (pg/mL)**			**Δ FSTL1 (ng/mL)**			**Δ Musclin (pg/mL)**			**Δ FGF21 (ng/mL)**			**Δ Leptin (µg/mL)**		
	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P
Model 1 (Simple regression)																								
BAT volume (mL)	1.971	0.007	0.657	0.748	0.007	0.642	-0.002	0.009	0.620	-4.656	0.043	0.379	0.001	0.011	0.599	0.078	0.020	0.461	-0.24	0.002	0.840	-5.296	0.019	0.457
BAT SUVmean	0.027	0.001	0.885	0.082	0.048	0.226	<0.001	0.012	0.567	-0.301	0.102	0.170	<0.001	0.003	0.789	<0.001	<0.001	0.981	-0.005	0.033	0.355	-0.247	0.023	0.412
BAT SUVpeak	0.404	0.007	0.641	0.514	0.090	0.095	-0.001	0.045	0.269	-0.874	0.038	0.413	<0.001	0.001	0.887	0.004	0.002	0.835	-0.021	0.030	0.376	-0.919	0.015	0.509
BAT radiodensity (HU)	0.258	0.003	0.816	**1.050**	**0.258**	**0.011**	-0.001	0.067	0.257	-1.856	0.061	0.395	<0.001	0.018	0.566	0.015	0.022	0.502	0.001	0.001	0.978	0.002	-0.373	0.850
Model 2 (Date PET-CT)																								
BAT volume (mL)	2.587	0.150	0.537	1.143	0.155	0.454	-0.005	0.171	0.316	-5.707	0.233	0.249	<0.001	0.124	0.756	0.135	0.236	0.170	-0.033	0.163	0.766	-7.171	0.183	0.285
BAT SUVmean	0.054	0.149	0.761	0.099	0.218	0.116	<0.001	0.187	0.266	-0.340	0.247	0.107	<0.001	0.143	0.986	0.002	0.186	0.580	-0.005	0.206	0.279	-0.329	0.198	0.243
BAT SUVpeak	0.537	0.180	0.505	**0.604**	**0.538**	**0.033**	-0.002	0.244	0.084	0.204	0.161	0.315	<0.001	0.131	0.918	0.015	0.214	0.425	-0.023	0.186	0.305	-1.304	0.196	0.315
BAT radiodensity (HU)	-0.332	0.009	0.773	**1.068**	**0.520**	**0.012**	-0.001	0.077	0.301	-0.031	0.062	0.545	<0.001	0.026	0.517	0.013	0.027	0.612	0.005	0.041	0.861	-0.314	0.016	0.876
Model 3 (BMI)																								

BAT volume (mL)	1.807	0.008	0.692	0.704	0.009	0.669	0.002	0.023	0.460	-3.596	0.223	0.466	0.001	0.011	0.606	0.079	0.023	0.464	-0.051	0.077	0.671	-5.636	0.019	0.498
BAT SUVmean	0.040	0.007	0.834	0.086	0.058	0.213	<0.001	0.015	0.607	-0.280	0.143	0.206	<0.001	0.016	0.771	<0.001	0.005	0.989	-0.005	0.033	0.366	-0.403	0.052	0.249
BAT SUVpeak	0.423	0.008	0.636	0.522	0.092	0.098	<0.001	0.018	0.489	-0.746	0.102	0.484	<0.001	0.006	0.875	0.004	0.002	0.839	-0.023	0.045	0.337	-1.272	0.022	0.434
BAT radiodensity (HU)	0.367	0.016	0.750	**1.039**	**0.260**	**0.015**	<0.001	0.021	0.940	-1.857	0.061	0.416	<0.001	0.034	0.588	0.016	0.045	0.483	0.007	0.036	0.817	-1.475	0.040	0.530
Model 4 (Baseline levels)																								
BAT volume (mL)	7.124	0.089	0.192	1.922	0.089	0.271	<0.001	0.020	0.550	-3.305	0.142	0.528	<0.001	0.047	0.875	0.071	0.020	0.630	-0.014	0.010	0.910	-4.737	0.020	0.607
BAT SUVmean	0.154	0.029	0.513	0.124	0.109	0.094	<0.001	0.016	0.516	-0.240	0.216	0.260	<0.001	0.021	0.631	-0.004	0.030	0.548	-0.004	0.044	0.420	-0.146	0.030	0.707
BAT SUVpeak	1.139	0.051	0.293	0.768	0.190	0.022	0.001	0.041	0.306	-0.587	0.147	0.575	<0.001	0.043	0.622	0.002	0.002	0.937	-0.018	0.045	0.451	-0.016	0.039	0.993
BAT radiodensity (HU)	-0.248	0.014	0.872	**1.264**	**0.327**	**0.005**	<0.001	0.003	0.893	-2.616	0.231	0.228	<0.001	0.072	0.371	0.005	0.026	0.897	-0.001	0.042	0.961	-1.941	0.038	0.474

Delta (Δ) of norepinephrine, lactate, BDNF, IL6 and FSTL1 were calculated by subtracting the baseline concentration to the circulating levels obtained 3 minutes after exercise. Delta (Δ) of musclin and leptin was calculated by subtracting the baseline concentration to the circulating levels obtained 30 minutes after exercise. Delta (Δ) of FGF21 was calculated by subtracting the baseline concentration to the circulating levels obtained 60 minutes after exercise. Unstandardized β, R^2, and P from simple and multiple linear regressions. Model 1: Simple regression. Model 2: Model 1 adjusted for the date when positron emission tomography/computed tomography assessment was performed. Model 3: Model 1 adjusted for BMI. Model 4: Model 1 adjusted for baseline level of the exerkine. Abbreviations: BAT: brown adipose tissue; BDNF: brain-derived neurotrophic factor; FGF21: fibroblast growth factor 21; HU: Hounsfield units; PET-TC: positron emission tomography/computed tomography; SUV: Standardized uptake value.

Table 17 shows the associations of endurance exercise-induced changes in circulating concentrations of exerkines with body composition, cardiometabolic risk factors, and physical fitness. The exercise-induced decrease in leptin concentration was negatively associated with BMI (β=-1.441, R^2=0.189, P=0.011), fat mass (β=-3.882 R^2=0.274, P=0.002), VAT mass (β=-57.063, R^2=0.147, P=0.028), triacylglycerols (β=-20.881, R^2=0.115, P=0.040) and diastolic blood pressure (β=-2.831, R^2=0.153, P=0.020). However, these associations disappeared after adjusting for baseline leptin concentrations. The log2 exercise-induced increase in BDNF concentration was also negatively associated with BMI (β=-5.426 R^2=0.178, P=0.018), HOMA-IR (β=-1.559 R^2=0.244, P=0.003), insulin (β=-6.263 R^2=0.229, P=0.004), glucose (β=-9.513 R^2=0.267, P=0.001), waist circumference (β=-14.990 R^2=0.161, P=0.025), and fat mass (β=-13.256 R^2=0.226, P=0.007) (data not shown). These associations persisted after adjusting for baseline plasma BDNF concentration. None of the exercise-induced changes in the concentration of the other exerkines was associated with body composition parameters, cardiometabolic risk factors or physical fitness (**Table 17**). The association among the exercise-induced changes in circulating exerkines assessed in the *confirmatory study* is shown in **Table 18**.

Table 17. Associations between endurance exercise-induced changes in plasma levels of exerkines and body composition, cardiometabolic risk factors and physical fitness (n=38).

	Δ Norepinephrine (ng/mL)			Δ Lactate (mmol/L)			Δ BDNF (ng/mL)			Δ IL6 (pg/mL)			Δ FSTL1 (ng/mL)			Δ Musclin (pg/mL)			Δ FGF21 (ng/mL)			Δ Leptin (µg/mL)		
	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P	β	R^2	P
Body composition																								
BMI (kg/m^2)	0.481	0.061	0.159	0.031	0.015	0.484	-0.001	0.056	0.201	0.021	<0.001	0.956	<0.001	0.005	0.708	-0.002	0.002	0.822	0.006	0.015	0.519	-1.441	0.189	**0.011***
WC (cm)	1.092	0.039	0.266	0.102	0.029	0.335	-0.002	0.061	0.182	0.562	0.020	0.532	<0.001	0.024	0.412	-0.033	0.054	0.200	-0.002	<0.001	0.939	-3.050	0.104	0.067
Lean mass (kg)	0.770	0.039	0.261	0.092	0.045	0.229	-0.756	0.034	0.319	-0.851	0.075	0.216	-0.140	0.012	0.563	0.012	0.017	0.479	0.006	0.004	0.738	-1.381	0.046	0.228
Fat mass (kg)	0.111	0.065	0.144	0.020	0.002	0.808	-1.240	0.067	0.161	0.686	0.042	0.362	0.108	0.005	0.697	0.004	0.002	0.807	0.020	0.037	0.309	-3.882	0.274	**0.002***
Fat mass %	0.652	0.020	0.430	-0.051	0.011	0.555	-0.001	0.015	0.505	1.113	0.097	0.158	<0.001	0.015	0.520	0.010	0.008	0.619	0.017	0.023	0.426	-2.382	0.101	0.071
VAT mass (g)	17.410	0.040	0.259	1.055	0.013	0.528	-0.025	0.062	0.178	10.121	0.021	0.516	0.003	0.007	0.652	-0.107	0.002	0.793	0.188	0.007	0.651	-57.063	0.147	**0.028***
Cardiometabolic risk factors																								
Glucose (mg/dL)	0.853	0.079	0.087	0.061	0.029	0.309	-0.001	0.074	0.113	-0.531	0.052	0.273	<0.001	0.002	0.812	-0.006	0.011	0.549	0.019	0.054	0.187	-0.423	0.007	0.632
Insulin (µUl/mL)	0.362	0.027	0.320	0.032	0.012	0.507	-0.001	0.070	0.125	0.352	0.088	0.149	<0.001	0.003	0.768	-0.003	0.007	0.624	0.016	0.082	0.102	-0.441	0.014	0.481
HOMA-IR	0.091	0.029	0.311	0.013	0.014	0.472	<0.001	0.081	0.097	0.071	0.067	0.211	<0.001	0.002	0.797	-0.001	0.007	0.625	0.004	0.082	0.100	-0.092	0.013	0.510
Triglycerides (mg/dL)	5.770	0.025	0.345	-0.183	0.002	0.781	-0.007	0.036	0.273	-0.053	<0.001	0.992	0.001	0.001	0.743	-0.073	0.011	0.536	0.112	0.023	0.391	-20.881	0.115	**0.040***
Total Cholesterol (mg/dl)	0.442	<0.001	0.904	0.332	0.019	0.407	-0.001	0.001	0.825	4.654	0.070	0.200	0.001	0.035	0.289	0.037	0.008	0.603	0.147	0.067	0.140	0.141	<0.001	0.982
HDL-C (mg/dL)	-0.243	0.002	0.777	0.051	0.007	0.608	<0.001	<0.001	0.919	0.212	0.003	0.803	<0.001	0.012	0.542	-0.009	0.009	0.593	0.005	0.001	0.846	0.012	<0.001	0.994
LDL-C (mg/dL)	-0.254	<0.001	0.930	0.362	0.038	0.240	-0.001	0.003	0.739	4.331	0.105	0.114	0.001	0.047	0.219	0.047	0.022	0.383	0.116	0.074	0.120	-0.801	0.001	0.871
Systolic BP (mm Hg)	1.392	0.056	0.166	0.172	0.083	0.088	-0.001	0.043	0.232	-1.051	0.055	0.272	<0.001	0.004	0.740	-0.006	0.003	0.741	0.004	<0.001	0.921	-1.930	0.040	0.246
Diastolic BP (mm Hg)	-0.343	0.006	0.654	0.013	0.001	0.852	<0.001	0.004	0.736	-1.012	0.073	0.203	<0.001	0.005	0.711	0.001	<0.001	0.923	0.027	0.021	0.433	-2.831	0.153	**0.020***
Physical fitness																								
Time exhaustion (min)	-0.112	0.003	0.747	0.003	<0.001	0.936	<0.001	0.002	0.789	0.051	0.008	0.601	<0.001	<0.001	0.970	0.009	0.056	0.157	0.002	0.001	0.871	0.063	<0.001	0.915
VO_2 max (mL/kg/min)	-0.683	0.030	0.321	-0.032	<0.001	0.911	0.001	0.058	0.163	-0.841	0.098	0.076	<0.001	0.004	0.708	-0.008	0.011	0.544	-0.008	0.007	0.657	1.511	0.050	0.196

Handgrip strength (kg)	0.324	0.008	0.621	-0.311	0.053	0.196	-0.001	0.065	0.152	-0.430	0.032	0.334	<0.001	0.003	0.763	0.022	0.097	0.073	-0.004	0.002	0.834	-0.122	<0.001	0.913
RM leg press (kg)	4.642	0.032	0.319	-1.521	0.025	0.376	-0.006	0.047	0.225	-5.031	0.080	0.123	-0.001	0.004	0.720	0.056	0.011	0.547	0.111	0.030	0.362	-6.312	0.020	0.428
RM bench press (kg)	0.972	0.044	0.241	-0.382	0.050	0.210	-0.001	0.026	0.373	-0.672	0.040	0.278	<0.001	<0.001	0.909	0.010	0.010	0.570	-0.004	0.001	0.873	-0.811	0.011	0.570

Delta (Δ) of norepinephrine, lactate, BDNF, IL6 and FSTL1 were calculated by subtracting the baseline concentration to the circulating levels obtained 3 minutes after exercise. Delta (Δ) of musclin and leptin was calculated by subtracting the baseline concentration to the circulating levels obtained 30 minutes after exercise. Delta (Δ) of FGF21 was calculated by subtracting the baseline concentration to the circulating levels obtained 60 minutes after exercise. Unstandardized β, R^2, and P from simple linear regressions. *Associations disappeared after adjusting for the baseline level of the exerkine. Abbreviations: BDNF: brain-derived neurotrophic factor; BMI: body mass index; BP: blood pressure; FGF21: fibroblast growth factor 21; HDL-C: high density lipoproteins cholesterol; LDL-C: low density lipoproteins cholesterol; RM: 1 Repetition Maximum. VAT: visceral adipose tissue; VO2 max; WC: waist circumference.

Table 18. Correlation matrix between the exercise-induced changes in circulating exerkines (confirmatory study n=38).

		Δ Leptin	Δ Norepinephrine	Δ IL6	Δ Lactate	Δ FSTL1	Δ Musclin	Δ FGF21	Δ BDNF
Δ Leptin	r		-0.058	-0.032	0.015	-0.040	0.075	-0.359	-0.114
	P		0.804	0.891	0.947	0.864	0.746	0.110	0.623
Δ Norepinephrine	r	-0.058		0.118	0.446	0.085	-0.075	0.335	0.001
	P	0.804		0.610	**0.043**	0.715	0.748	0.138	0.996
Δ IL6	r	-0.032	0.118		-0.020	0.411	0.158	0.485	0.406
	P	0.891	0.610		0.932	0.064	0.493	**0.026**	0.068
Δ Lactate	r	0.015	0.446	-0.020		0.048	-0.027	-0.106	0.022
	P	0.947	**0.043**	0.932		0.836	0.906	0.648	0.923
Δ FSTL1	r	-0.040	0.085	0.411	0.048		0.351	0.383	0.629
	P	0.864	0.715	0.064	0.836		0.119	0.087	**0.002**
Δ Musclin	r	0.075	-0.075	0.158	-0.027	0.351		-0.031	-0.092
	P	0.746	0.748	0.493	0.906	0.119		0.895	0.692
Δ FGF21	r	-0.359	0.335	0.485	-0.106	0.383	-0.031		0.195
	P	0.110	0.138	**0.026**	0.648	0.087	0.895		0.397
Δ BDNF	r	-0.114	0.001	0.406	0.022	0.629	-0.092	0.195	
	P	0.623	0.996	0.068	0.923	**0.002**	0.692	0.397	

R and P values from Pearson correlation analyses. Abbreviations: BDNF: brain-derived neurotrophic factor; FGF21: fibroblast growth factor 21. Delta (Δ) of norepinephrine, lactate, BDNF, IL6 and FSTL1 were calculated by subtracting the baseline concentration to the circulating levels obtained 3 minutes after exercise. Delta (Δ) of musclin and leptin was calculated by subtracting the baseline concentration to the circulating levels obtained 30 minutes after exercise. Delta (Δ) of FGF21 was calculated by subtracting the baseline concentration to the circulating levels obtained 60 minutes after exercise.

DISCUSSION

In the present study, we investigated, in young sedentary adults, the acute and chronic effect of exercise on plasma levels of 16 exerkines that have been shown to regulate BAT metabolism and/or WAT browning in preclinical models. We found that a maximum walking effort test on a treadmill increased the circulating concentration of BAIBA, lactate, norepinephrine, BDNF, IL6, FSTL1, musclin, and FGF21, reduced leptin concentration, and did not modify the levels of adiponectin, ANP, meteorin-like and follistatin. Plasma levels of pro-ANP, myostatin, and irisin were not detected in more than half of the individuals. Interestingly, the exercise-induced change in lactate concentration was positively associated with BAT volume, ^{18}F-FDG uptake and radiodensity. A bout of resistance exercise increased lactate levels, without affecting the rest of analysed exerkines. Finally, we did not observe any long-term effect of an exercise training program including endurance and resistance exercise on the plasma levels of the studied exerkines.

The changes observed after acute endurance exercise in the circulating concentrations of leptin, lactate, norepinephrine, BDNF, IL6, FSTL1, and FGF21, are consistent with previous studies' results [105]. However, this is the first study reporting an increase in plasma levels of musclin after a bout of exercise in humans. Stautemas et al. previously reported an increase in BAIBA plasma levels after 1 hour of an incremental cycling test in trained subjects [307], whereas Morales et al did not observe any effect in untrained individuals [308]. Although we found an increase in plasma BAIBA levels in our *discovery study*, it should be noted that BAIBA concentrations were detected only in 60% of the participants. The differences observed between our study and others might be explained by the different training status of participants, as well as the duration and intensity of exercise. In our study, untrained individuals performed a short but very intense session of exercise.

In our study, a maximum effort test on a treadmill did not alter the plasma levels of adiponectin, ANP, meteorin-like or follistatin, which have been shown to produce WAT browning in preclinical models [105]. Previous studies in middle-aged and young adults observed increases in plasma levels of ANP [190], follistatin [344], and meteorin-like [211] after a session of moderate-intensity endurance exercise between 40 and 60 minutes of duration. These findings suggest that a lower intensity and greater duration might be required to stimulate these exerkines secretion. Moreover, increases in follistatin concentration have been reported after 3 h of exercise [344], whereas the last blood sample in our study was collected 2 h after the end of the exercise bout. Previous studies have also reported controversial results for the acute effect of endurance exercise on circulating adiponectin [269,401], myostatin [344], and irisin [105,344]. Furthermore, irisin results should be considered with caution since the detectability of irisin in humans has been questioned [196]. Overall, exercise

characteristics such as intensity or duration, or characteristics of the study subject, such as age or training status, are likely explaining part of these controversies. It also should be noted that the exercise-induced changes exhibited different kinetics for some of the analysed exerkines. This may be due to the diverse nature of the molecules. While some of them are end products of metabolic pathways, such as lactate, others, like FGF21, are proteins resulting from the complex processes of transcription and translation, which indeed takes time.

We observed an intriguing and consistent positive association between the exercise-induced change in lactate concentrations and BAT volume, ^{18}F-FDG uptake and radiodensity. Lactate is a product of anaerobic glycolysis, whose secretion is related with the exercise intensity and type IIb muscle fibre proportion [315]. Murine brown adipocytes internalize lactate in response to exercise, leading to an increase in UCP1 thermogenic activity and UCP1 and FGF21 expression in adipocytes, inducing WAT browning [402]. Thus, the association found in our study might reflect the existence of a muscle-BAT crosstalk taking place during exercise. Indeed, other molecules have been previously shown to be part of this bidirectional inter-organ communication [95,96]. The relation between BAT function and skeletal muscle composition and function deserves further investigation.

We found a negative association between the acute endurance exercise-induced change in BDNF plasma levels and BMI, HOMA-IR, glucose, insulin, waist circumference, and fat mass. It suggests that participants with a fatter and less favourable metabolic profile have a smaller increase in BDNF after endurance exercise than their counterparts. These results are in line with those obtained by Roth et al [403], who observed unchanged serum BDNF levels after one year of exercise intervention in children with obesity, but not in children with normal-weight. Other authors have reported negative associations between serum BDNF levels and BMI [404], which is in line with our results. We also observed a negatively association between exercise-induced decrease in leptin concentration and BMI, fat mass, VAT mass, triacylglycerols and diastolic blood pressure. However, these associations disappeared after adjusting for baseline leptin concentrations, suggesting that these parameters are associated with baseline leptin plasma levels instead of with the exercise-induced change [405].

We also explored the effect of resistance exercise on the selected exerkines. We only observed an increase of plasma levels of lactate after the bout of resistance exercise, which is in line with results previously observed [315]. It should be remarked that the increase observed in plasma levels of lactate after the maximum effort test was twice the observed in the session of acute resistance exercise, probably due to the difference in intensity (2.2 mmol/L vs. 4.6 mmol/L, P=0.021).

Previous studies also failed to observe any effect of resistance exercise on circulating concentrations of ANP [341], myostatin [368], FGF21 [356], and BDNF [261]. Other exerkines, such as meteorin-like, musclin, FSTL1, adiponectin, and BAIBA, which we found not to be modified, have not been analysed in humans in response to resistance exercise. On the other hand, other studies have reported increases in norepinephrine [335], IL6 [356], and decreases in leptin concentrations [386] immediately and 30 minutes after resistance exercise. Importantly, the lack of effect observed in our study is likely explained, at least in part, by the training load imposed by the experimental session. The resistance exercise session was designed with the primary aim of assessing muscle strength, and thus, it was characterized by large recovery periods and relatively low training volume. New studies with more intense and denser resistance exercise sessions are needed to explore the effect of resistance exercise on these exerkines in humans.

Finally, we did not observe any effect of the 24-week training program on the resting circulating concentrations of exerkines. Some studies have reported an increase in circulating concentrations of some exerkines, such as adiponectin [275], BDNF [406], FGF21 [407], follistatin [239], and a decrease in myostatin [239] and leptin concentrations [408]. On the one hand, Racil et al observed an increase in plasma adiponectin levels in adolescent girls with obesity after 12 weeks of interval training [275]. Similarly, Pereira et al reported an increase in plasma BDNF levels in elderly women after 10 weeks of strength-training program [406]. Regarding FGF21, Keihanian et al. also observed an increase in serum levels in males with type-2 diabetes after a 8-week training program (both resistance and aerobic exercise) [407]. In addition, Bagheri et al. studied a 8-week training program combining resistance and aerobic exercise in 30 sarcopenic elderly men [239], observing an increase in serum follistatin levels and a decrease in serum myostatin levels. Finally, other studies have reported a decrease in plasma and serum leptin levels after chronic exercise in overweight and obese individuals, although it seems to be fat mass dependent [408]. The differences with the results obtained in our study might be explained by the health status and age of the individuals as well as the intensity and the duration of the training program.

Limitations

The findings of this study should be considered with caution, as some limitations are present. The importance of including sex as a biological variable is widely recognized and it is plausible that the effect of exercise on BAT-related exerkines differs in men and women [409,410]. However, our study was not designed for studying the effect of sex, and lack the statistical power to adequately tested sex differences. In addition, participants did not follow a familiarization period before the muscle strength measurements. The endurance and resistance sessions consist of relatively low training load and thereby, the stimulus could have not been strong enough to trigger a significant release of the aforementioned exerkines to the bloodstream. Furthermore,

despite the ^{18}F-FDG PET/CT is currently the best available method to assess BAT volume, it presents serious limitations for assessing its thermogenic activity [411].

Conclusion

In conclusion, we found that a short and intense endurance exercise bout increases blood concentrations of norepinephrine, lactate, BDNF, IL6, FSTL1, musclin, and FGF21, whereas it decreases leptin concentration. In contrast, the concentration of adiponectin, ANP, meteorin-like and follistatin was not modified by the endurance exercise bout. A bout of resistance exercise only increased the plasma levels of lactate immediately after resistance exercise. On the other hand, neither a low-volume resistance exercise session nor a 24-week exercise training program including both endurance and resistance training did modify the concentration of these endocrine signalling molecules. Altogether, our results show that a short and intense endurance exercise bout increases the plasma levels of several exerkines that could regulate BAT metabolism and WAT browning in sedentary young adults, yet only the exercise-induced change in lactate concentrations was associated with BAT volume, glucose uptake and radiodensity.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Since the activity of BAT in humans was recognized in 2009[1–4], its study and use as a potential therapeutic strategy in metabolic diseases has been gaining interest. BAT is characterized by its fundamental role in heat production through the uncoupling of oxidative phosphorylation in mitochondria[5]. Thermogenesis in brown adipocytes is due to the expression of UCP-1 protein in the mitochondrial inner membrane, dissipating energy as heat[6]. In order to perform thermogenesis, brown adipocytes have to consume substrates, mainly fatty acids[7–10], so it was thought that BAT could be a suitable therapeutic target against obesity by increasing energy expenditure and the clearance of fatty acids in the blood, among other molecules. Nevertheless, several studies have proved that the energy expenditure of BAT in humans is considerably less than in rodents, where most of the studies have been carried out[11]. Therefore, the beneficial effect of BAT must be attributable to other intrinsic characteristic of the tissue.

Current findings have demonstrated the ability of BAT to secrete and release molecules capable of acting at an autocrine, paracrine and endocrine level, the so-called batokines or brown adipokines[12–14]. These molecules can therefore act locally, enhancing tissue activity, or acting on distant organs, as it is exposed in **Chapter 1.1**. It is noteworthy to emphasize the recent endocrine role assigned to BAT, being able to release molecules into the bloodstream. In this context, some molecules have been identified in murine models to be released by BAT after activation, which has been attributable to beneficial effects on metabolism. Some of these actions are related to improved glucose or lipid metabolism, insulin sensitivity, cardioprotective effects or even enhancement of the WAT browning process, in which white adipocytes acquire a more brown-like phenotype by expressing the UCP-1 protein. Despite the great therapeutic potential assigned to BAT as an endocrine organ, its discovery is very incipient. In addition, the detection and monitoring of BAT activity in humans are very challenging, which have resulted in most of the available studies being conducted in mice or *in vitro* models, making difficult to extrapolate the findings obtained to human metabolism. It should also be noted that the translation of knowledge between BAT of mice and BAT of humans is quite complex. Therefore, further research in humans is warranted to identify potential batokines as well as their effects on the population.

Since most of the batokines have been recognized in mice, one of the objectives of this Doctoral Thesis is to identify 5 batokines (i.e., CXCL14, GDF15, FGF21, IL-6 and BMP8b) in young sedentary adults. For this purpose, participants were exposed to an individualized 2-hour cooling protocol, in which their shivering threshold had been previously determined. During this protocol, participants underwent ^{18}F-FDG PET-CT and three blood draws were taken (before the protocol and 1 and 2 hours after the start of the protocol). These results are exposed and discussed in **Chapter 1.2**, in **Study I (Figure 19).** We observed that cold exposure increased plasma levels of

CXCL14, GDF15, FGF21 and IL-6 while reducing BMP8b levels. In order to elucidate whether BAT might have been involved in the modulation of the plasma levels of these molecules, linear associations were obtained between the cold-induced changes in plasma levels and variables of BAT function (volume, ^{18}F-FDG uptake and radiodensity). We found that only the cold-induced changes in circulating levels of FGF21 were positively associated with BAT volume, and persisted after adjusting for PET-CT date, sex, and BMI. Although FGF21 is also produced and released by other tissues, such as the liver[15], these findings indicate that BAT might contribute to the increase in circulating levels of this molecule. Thus, and whilst further studies in humans are required, this work suggests that FGF21 could be a potential human batokine. In spite of not having reported an association between cold-induced changes in circulating levels of the analyzed molecules (CXCL14, GDF15, IL-6, and BMP8b) and BAT parameters in our study, we cannot deny that BAT is a possible source of the expression of these molecules. One of the underlying causes is the existence of methodological limitations of ^{18}F-FDG PET-CT, even though it is considered currently the gold-standard. An example of these limitations is the use of glucose as a radiotracer, despite the fact that the predominant substrate is known to be fatty acids. Hence, it is possible that the tissue activity is being underestimated, in addition to other limitations such as the staticity of the ^{18}F-FDG PET-CT used in our study. Furthermore, it is conceivable that small deposits of BAT or beige tissue are not being taken into account. These methodological limitations of the study are detailed in the last part of this section.

In summary, the Study I provides scientific evidence of the endocrine role of BAT in humans by identifying some batokines previously reported in mice, such as CXCL14, GDF15, FGF21, and IL-6. Furthermore, it reports a positive association between FGF21 levels and BAT volume, suggesting that, indeed, human BAT may be responsible for producing and releasing this molecule into the bloodstream. This is the first study to include such a high number of batokines measured in a population of humans after individualized cold exposure and together with a determination of BAT by ^{18}F-FDG PET-CT. This may be a starting point for future studies that focus on determining whether human BAT, after activation, is indeed the main source of these molecules in the bloodstream, as well as target tissues and metabolic effects. It would also be worthwhile to examine the endocrine role of BAT in different populations, such as the elderly, people with metabolic diseases, children, or the involvement of the menstrual cycle in BAT activity.

Future studies should additionally include the determination of other batokines, such as 12,13-diHOME, neurogulin-4, 12-HEPE, EDPR-1, or microRNAs, taking blood samples at different time intervals, modifying the duration of different cooling protocols as well as performing a dynamic PET-CT. With the development of these studies, the endocrine role of BAT

in humans could be elucidated. The identified batokines could be used as therapeutic strategies and as a method of prevention of cardiometabolic diseases.

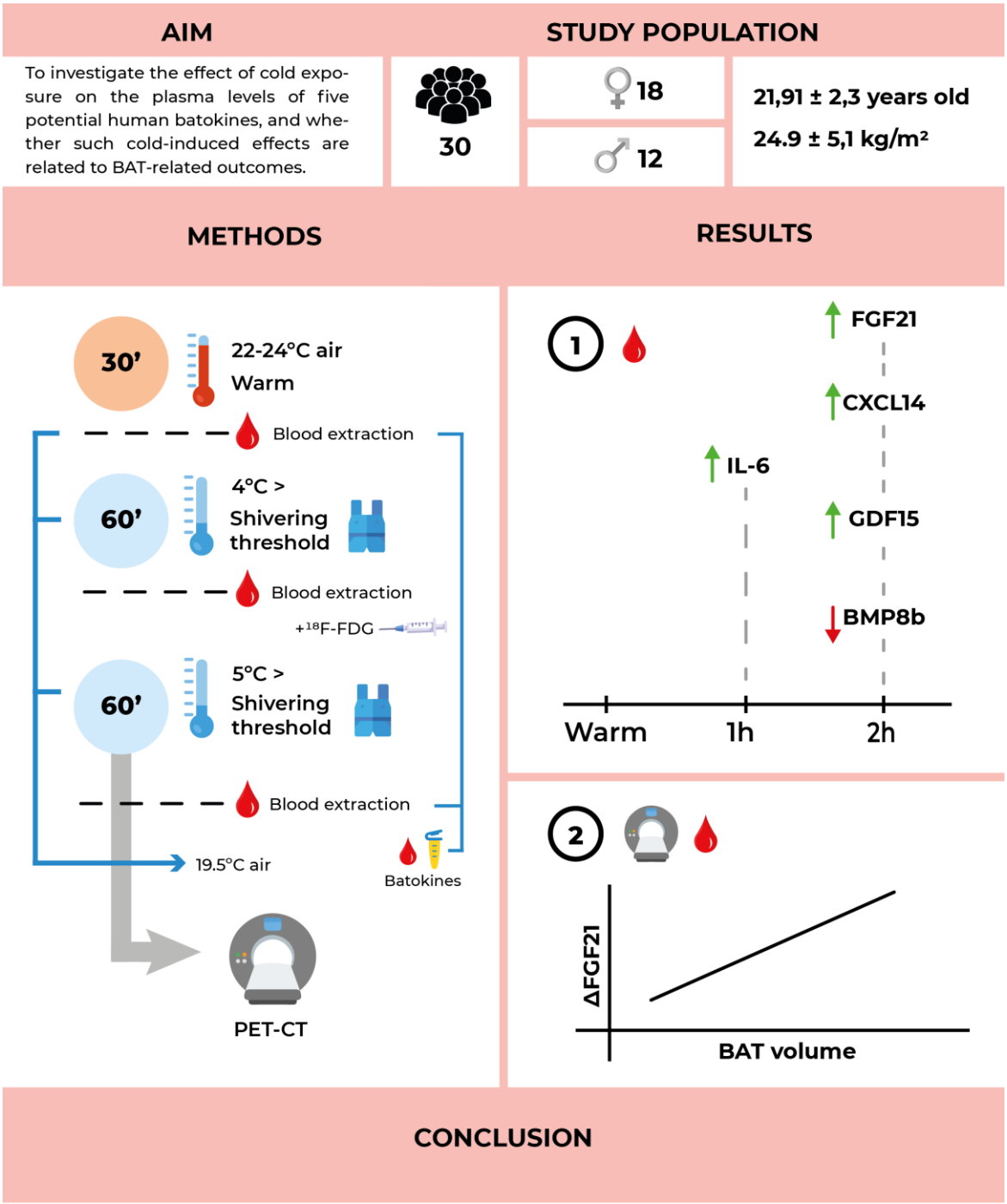


Figure 19. *Graphical summary of the Chapter I.*

This Doctoral Thesis pursues to understand the communication between the BAT and the rest of the tissues, studying the BAT not only as a producer of endocrine factors but also as a receptor of them. In this sense, **Chapter 2.2** aims to identify those endocrine factors released into the bloodstream after exercise, which could be activators of BAT or WAT browning. Given the multitude of metabolic benefits associated with BAT activity, different research groups are currently pursuing the goal of discovering different ways to activate the tissue without resorting to cold activation. In this aspect, and based on studies performed in murine models, exercise seems to be one of these BAT modulators[16,17]. One of the mechanisms by which exercise is thought to induce BAT activity or WAT browning in mice is via the release of endocrine factors, the so-called exerkines, reviewed in **Chapter 2.1.** To address the lack of studies in humans, the objective of identifying human exerkines and relating them to BAT function is addressed in **Chapter 2.2**, in **Study II (Figure 20)**. In this study, circulating levels of 16 exerkines are determined after three different types of exercise: i) acute endurance exercise, ii) acute resistance exercise and iii) a 24-week exercise program in young sedentary adults.

We observed that acute endurance exercise elevated plasma levels of lactate, norepinephrine, BDNF, IL-6, and follistatin-like 1 3 minutes after exercise, and musclin and FGF21 30 and 60 minutes after exercise, respectively. Acute endurance exercise also decreased plasma levels of leptin 30 minutes after exercise. Levels of adiponectin, ANP, BAIBA, meteorin-like, follistatin, pro-ANP, irisin, and myostatin were unchanged or undetectable. Acute resistance exercise only increased plasma lactate levels just after exercise and chronic exercise did not modify plasma levels of any exerkines. Although the results obtained in acute endurance exercise are consistent with previous findings, this is the first study to report an increase in circulating musclin levels in humans. The failure to see an increase in the rest of the exerkines may be due to different factors. For example, increments in circulating follistatin levels have been observed at 3 hours of exercise[18], whereas in our study the last recorded intake was at 2 hours. The chosen exerkines present different kinetics, so it would be desirable to carry out more studies that take blood samples at shorter intervals and for a longer period of time. In addition, the acute endurance exercise of the Study II consisted of a maximal effort test, so it is possible that the results might differ in other types of training at a lower intensity or longer duration. Regarding resistance exercise, it should be considered that the study was designed to assess the muscular strength of the participants, and therefore, the training volume was low. Further studies evaluating the effect of different types of resistance training on circulating levels of exerkines should therefore be considered. Finally, on the long-term effect of exercise, the literature is controversial[19–21]. The discrepancies observed may be due to the different health status of the population, the time elapsed between the end of the training program and the sampling, the age of the participants, etc.

The only exercise-induced change in circulating levels of exerkines related to BAT activity was lactate. We reported a consistent positive association between increased plasma lactate levels and BAT volume, ^{18}F-FDG uptake and radiodensity. Lactate has been reported to be internalized in murine brown adipocytes and enhance UCP-1 and FGF21 expression, leading to WAT browning[22]. It is relevant to emphasize that in the Study II, the maximum effort test occurs on a different day than the detection of BAT parameters. Therefore, although we cannot affirm that the BAT is a receptor of circulating lactate during or after exercise, we observed that in our population, those with higher BAT volume and activity have a greater increase in lactate after exercise. For this reason, these results could represent scientific evidence of a possible crosstalk between tissues, muscle and BAT. However, further human and *in vitro* studies are clearly needed to investigate the relationship between BAT and lactate in response to exercise.

In brief, the Study II of the present Doctoral Thesis provides information on understanding how human tissues communicate with BAT, in specifically, as a response to exercise. We reported a robust upregulation after acute endurance exercise of plasma lactate, norepinephrine, BDNF, IL-6, follistatin-like 1, musclin and FGF21 levels, as well as a decrease in leptin. Likewise, the increase in lactate was related to greater BAT activity and volume, which could be evidence of crosstalk between muscle and BAT. This study, which is the first to analyze a large number of exerkines, different types of exercise and accompanied by variables on BAT, may be the departure point for developing specific studies on the effect of each exerkine on BAT activity. Indeed, despite the observed increase in exerkines levels in our study, whether exercise activates BAT remains to be demonstrated. The literature is controversial and in fact, in our study, exerkines are not related to BAT activity or volume, with the exception of lactate. Thus, additional interventional human studies with large cohorts and well supervised exercise programs are essential to elucidate what type of exercise, intensity and duration, and in what population may have beneficial effects on BAT. Besides, in order to be able to determine if BAT is activated after exercise through exerkines, it would be appropriate to perform ^{18}F-FDG PET-CT, before and after exercise, accompanied by the exercise-induced plasma levels of several exerkines.

The two studies included in the Doctoral Thesis, taken together, reveal scientific evidence on the plausible communication between BAT and the rest of organs in human adults. Progress in this field is crucial in order to advance in the knowledge of human physiology and to offer a tool for the prevention and treatment of obesity and cardiometabolic diseases, improving the quality of life of people who suffer from it.

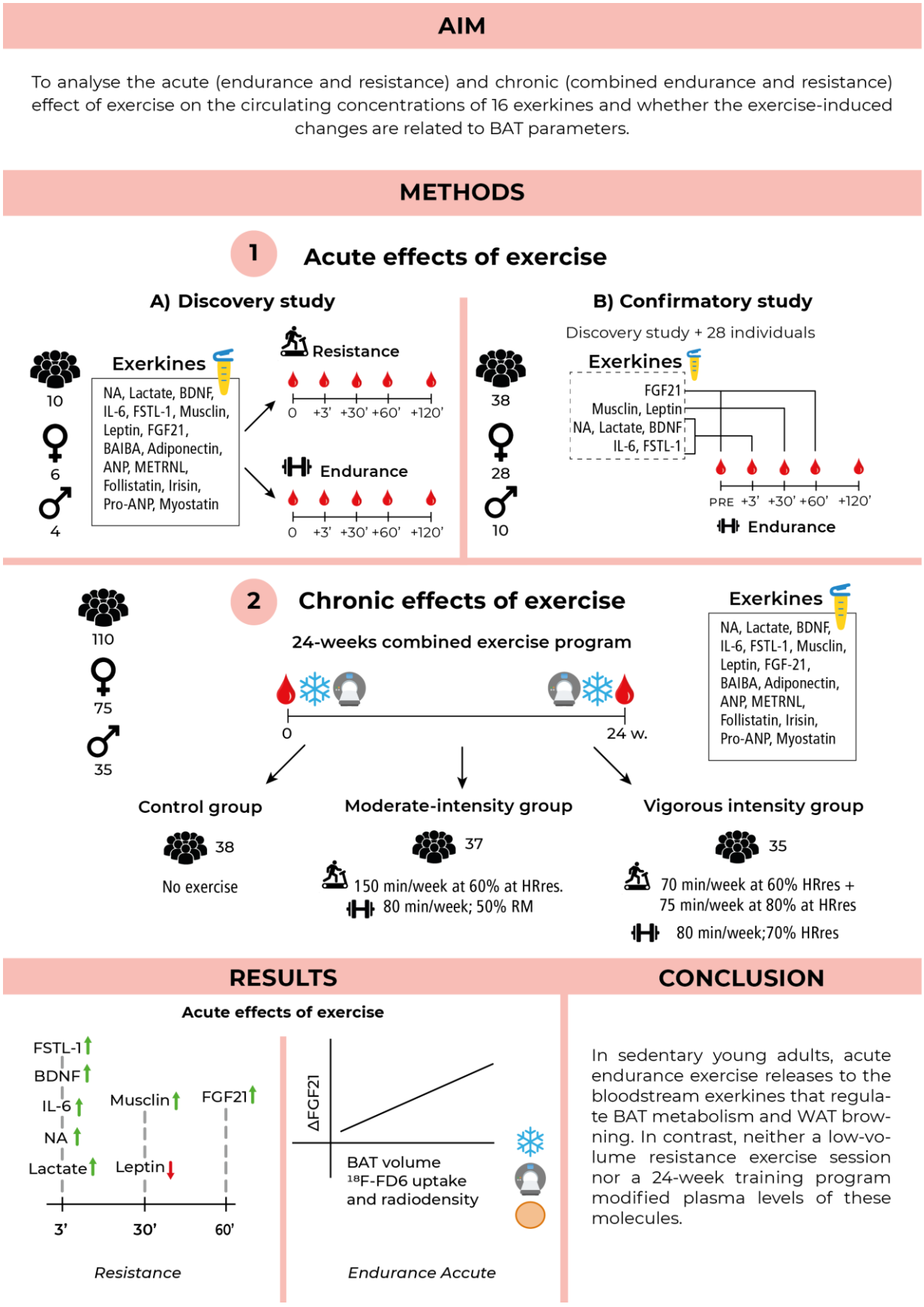


Figure 20. Graphical summary of the Chapter 2.

GENERAL LIMITATIONS

The results presented in this Doctoral Theses need to be interpreted with caution since there are some limitations:

- The studies included in the Present Doctoral Thesis have been performed in young, sedentary, and relatively healthy adults. So, the results presented are not extrapolatable to older populations or those with cardiometabolic disease.
- The absence of sex interaction in both studies should be considered with caution. This is due in first place, that the proportion of men in both studies were smaller than women and, in second place, that the menstrual cycle was not controlled or standardized, which may have affected the results.
- Exerkines plasma levels (Study II) and BAT ^{18}F-FDG activity were measured at different times of the day, so circadian rhythmicity could partially compromise the results of the Study II.
- Both studies examine the association of two parameters (e.g. BAT volume and plasma exerkines levels) performed in different days. Thus, an inter-day biological variability could have contributed to the lack of association.
- The anatomical regions analyzed by the PET-CT scan were from the cerebellum to thoracic vertebra 4. Despite these regions are supposed to cover the main BAT deposits[23], others like suprarenal BAT deposits, were excluded in both studies.
- In spite of being the best available technique for human BAT *in vivo* quantification[24,25], static ^{18}F-FDG PET-CT scan could not represent BAT thermogenesis[26,27]. BAT mainly fuel are triglycerides, which result from intracellular lipolysis and plasma and lipoproteins non esterified fatty acids[6,8,10,11]. However, the radiotracer used to estimate BAT metabolic activity in the ^{18}F-FDG PET-CT is a glucose analogue. If the present results would be replicated using other radiotracers such as 150-oxygen or 11C-acetate remains to be explored. Besides, PET image resolution could have underestimated BAT volume[25].
- We assessed BAT volume and activity using a static PET-CT scan, which only reflects the accumulated ^{18}F-FDG uptake over a 120 min period. The use of a dynamic PET-CT scan would allow to measure BAT activity at different time-points and detect associations between ^{18}F-FDG uptake during cold-stimulation changes in batokines (Study I).
- Regarding the Study II, and, specifically, the longitudinal study, measures of the participants were assessed in different moments of the year, contributing to increase the variance in the analyzed parameters. Therefore, although the control group and the homogenic distribution in the date of evaluation may avoid a nonsystematic error, it

cannot be discard that the exercise-related effect on BAT metabolism have been blunted by seasonality.

- We used an individualized cooling protocol based on the individuals' shivering threshold for BAT stimulation. Although this method is currently considered adequate and valid for BAT activation[24,28,29], it should be noted that we determined shivering through direct observation and also self-reported by participants, which may be not very objective. Additional studies should be conducted to compare the use of objective shivering threshold determination versus more objective methods such as electromyography.

CONCLUSIONS

CONCLUSIONS

General conclusion

In young adults, an individualized cooling protocol modulates the plasma levels of CXCL14, GDF15, FGF21, IL-6 and BMP8b, five batokines previously identified in rodents. Additionally, acute endurance exercise modulates the plasma levels of norepinephrine, lactate, BDNF, IL-6, FSTL1, musclin, FGF21, and leptin, exerkines that are suggested to modify BAT activity. Thus, the Present Doctoral Thesis provides findings that suggest that human BAT communicates with the rest of tissues through endocrine mechanisms.

Specific conclusions

Study I – Cold exposure modulates circulating batokines in humans, but only FGF21 is associated with brown adipose tissue volume

- A 2-hours personalized cold exposure increases the plasma levels of CXCL14, GDF15, FGF21 e IL-6 and decreases the plasma levels of BMP8b in young adults.
- The cold-induced increase in FGF21 plasma levels is positively associated with BAT volume, suggesting that human BAT might secrete this molecule to an extent able to impact to the circulating pool.

Study II – Exercise-induced changes on exerkines that might influence brown adipose tissue metabolism in young sedentary adults

- A short and intense endurance exercise bout increases blood concentrations of norepinephrine, lactate, BDNF, IL-6, FSTL1, musclin, and FGF21, whereas it decreases leptin concentration. In contrast, the concentration of adiponectin, ANP, meteorin-like and follistatin was not modified by the endurance exercise bout. A bout of resistance exercise only increased the plasma levels of lactate immediately after resistance exercise. On the other hand, 24-week exercise training program including both endurance and resistance training did modify the concentration of these endocrine signalling molecules.

- The endurance acute-induced exercise increase in plasma lactate levels was positively associated with BAT volume, glucose uptake and radiodensity, suggesting an inter-organ communication between BAT and skeletal muscle.

CONCLUSIONES

Conclusión general

En adultos jóvenes, un protocolo de enfriamiento individualizado modula los niveles plasmáticos de CXCL14, GDF15, FGF21, IL-6 y BMP8b, cinco batoquinas previamente identificadas en roedores. Además, el ejercicio agudo aeróbico modula los niveles plasmáticos de norepinefrina, lactato, BDNF, IL-6, FSTL1, musclin, FGF21 y leptina, exerquinas que podrían ser moduladoras de la actividad del TAP. Por lo tanto, la presente Tesis Doctoral aporta hallazgos que sugieren que el TAP humano se comunica con el resto de tejidos a través de mecanismos endocrinos.

Conclusiones específicas

Estudio I. La exposición al frío modula las batoquinas circulantes en humanos, pero sólo FGF21 se asocia con el volumen del tejido adiposo pardo (TAP).

- Una exposición personalizada al frío durante 2 horas aumenta los niveles plasmáticos de CXCL14, GDF15, FGF21 e IL-6 y disminuye los niveles plasmáticos de BMP8b en adultos jóvenes.
- El aumento inducido por el frío en los niveles plasmáticos de FGF21 se asocia positivamente con el volumen del TAP, lo que sugiere que el TAP humano podría secretar esta molécula en una medida capaz de repercutir en el pool circulante.

Estudio II - Cambios en las exerquinas inducidos por el ejercicio que podrían influir en el metabolismo del tejido adiposo pardo en adultos jóvenes sedentarios

- Una sesión corta e intensa de ejercicio aeróbico aumenta las concentraciones sanguíneas de norepinefrina, lactato, BDNF, IL-6, FSTL-1, musclin y FGF21, mientras que disminuye la concentración de leptina. En cambio, la concentración de adiponectina, ANP, meteorina-like y follistatina no se vio modificada por el ejercicio aeróbico. El ejercicio de resistencia sólo aumentó los niveles plasmáticos de lactato inmediatamente después del ejercicio. Por otro lado, un programa de entrenamiento de 24 semanas que incluía tanto ejercicios aeróbicos como de resistencia modificó la concentración de estas moléculas de señalización endocrina.
- El aumento de los niveles plasmáticos de lactato inducido por el ejercicio agudo aeróbico se asoció positivamente con el volumen del TAP, la captación de

glucosa y la radiodensidad, lo que sugiere una comunicación entre órganos entre el TAP y el músculo esquelético.

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