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Bone health in children with overweight/obesity: The role of systemic chronic inflammation, vitamin D and muscular fitness.

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Doctoral Programme in Biomedicine

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Salud ósea en niños con sobrepeso/obesidad: Rol de la inflamación sistémica crónica, la vitamina D y la fuerza muscular

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A los Gigantes, sin los cuales esta Tesis no existiría.

Especialmente a mi familia.

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1. RESEARCH PROJECT AND FUNDING

RESEARCH PROJECT AND FUNDING

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2. ABSTRACT/RESUMEN

Abstract

Background

Previous research suggests a link between obesity and bone morphogenesis which may be explained by inflammatory markers and adipokines. It is known that bone development is regulated by modelling and remodelling processes that depend on the mechanical forces applied by the muscles to the skeleton. Furthermore, the relationships of vitamin D and muscular strength with bone have been described, but no study has jointly examined the association of these predictors with bone.

Objectives

The present Doctoral Thesis aimed to study which inflammatory markers and adipokines are associated with bone health and whether these associations are modified by muscular fitness levels in children with overweight/obesity (**study 1 and study 2**)**.** In addition, this Thesis examined whether the relationship between vitamin D (i.e., plasma calcidiol) and bone health was mediated by muscular fitness (**study 3**)**.**

Methods

To address these aims, we used baseline data from the ActiveBrains project. Plasma interleukin (IL)-1β, IL-6, tumour necrosis factor-α (TNF-α), epidermal growth factor, vascular endothelial growth factor A (VEGF), C-reactive protein, leptin, adiponectin and calcidiol were analysed. Muscular fitness was assessed in laboratory (through determining each participant's 1 repetition maximum at bench and leg press) and in field-based conditions (handgrip strength and standing long jump). Bone outcomes, fat and lean mass were assessed by dual-energy x-ray absorptiometry. For **study 1 and**

study 2, the association between these biochemical markers and bone outcomes was tested with multiple regression analyses controlling for sex, somatic maturation and lean mass. Additionally, the muscular fitness interaction was examined in the associations of inflammatory markers and adipokines with bone outcomes. For **study 3,** the mediator role of muscular fitness was examined in the association between calcidiol and bone outcomes controlling for sex, somatic maturation, lean mass and season.

Results and main findings

In **study 1,** IL-6 and VEGF levels were negatively associated with bone mineral content at the total body, whereas TNF-α (negatively) and IL-1β (positively) levels were associated with bone mineral content at the lumbar spine. In **study 2,** no association was found between leptin or adiponectin levels and bone outcomes. Furthermore, high levels of objectively measured muscular fitness may attenuate the adverse effects of VEGF and TNF-α on bone mineral content at the total body and lumbar spine, respectively (**study 1**). In addition, a negative association between leptin and bone mineral content at the lumbar spine was observed in the low lower-body muscular fitness group, whereas no significant association was found in the high lower-body muscular fitness group (**study 2**). In **study 3,** calcidiol was indirectly associated with areal bone mineral density at the total body, arms and legs through relative muscular fitness.

Overall conclusions

The results of this Thesis suggest that IL-1β, IL-6, TNF-α, VEGF and leptin (to a lesser extent) could be contributing factors explaining the link between obesity and bone health in children with overweight/obesity. Optimal muscular fitness levels may attenuate the adverse effects of TNF-α, VEGF and leptin on bone. Moreover, muscular fitness seems to play a key role in the relationship of calcidiol levels and bone outcomes in this population. Altogether, appropriate levels of vitamin D and muscular fitness may preserve normal bone accretion in children with overweight/obesity.

Resumen

Antecedentes

Investigaciones previas sugieren un vínculo entre la obesidad y el desarrollo óseo, y este vínculo puede estar explicado por los marcadores de inflamación y las adipocinas. Se sabe que el desarrollo óseo también está regulado por procesos de modelado y remodelado, que dependen de las fuerzas mecánicas aplicadas por los músculos al esqueleto. Además, se han descrito previamente las relaciones de la vitamina D y la fuerza muscular con el hueso, pero ningún estudio ha examinado conjuntamente la asociación de estos predictores con el hueso.

Objetivos

La presente Tesis Doctoral tuvo como objetivo estudiar qué marcadores de inflamación y adipocinas se asocian con la salud ósea, y si estas asociaciones se ven modificadas por los niveles de fuerza muscular en niños con sobrepeso u obesidad (**estudio 1 y estudio 2**). Además, esta Tesis examinó si la relación entre la vitamina D (es decir, calcidiol en plasma) y la salud ósea estaba mediada por la fuerza muscular (**estudio 3**).

Métodos

Para abordar estos objetivos, utilizamos datos de la evaluación inicial del proyecto ActiveBrains. Se analizaron niveles plasmáticos de interleucina (IL)-1β, IL-6, factor de necrosis tumoural-α (TNF-α), factor de crecimiento epidérmico, factor de crecimiento endotelial vascular A (VEGF), proteína C reactiva, leptina, adiponectina y calcidiol. La fuerza muscular se evaluó en el laboratorio (determinando la 1RM de cada

participante en press de banca y press de piernas) y en condiciones de campo (fuerza de agarre y salto de longitud desde parado). Los resultados óseos, la grasa y la masa magra se evaluaron mediante absorciometría de rayos X de energía dual. Para el **estudio 1** y el **estudio 2**, la asociación entre estos marcadores bioquímicos y los resultados óseos se verificó mediante análisis de regresión múltiple que controlan el sexo, la maduración somática y la masa magra. Además, se examinó la interacción de la fuerza muscular en las asociaciones de los marcadores bioquímicos con los resultados óseos. Para el **estudio 3**, se examinó el papel mediador de la fuerza muscular en la asociación entre el calcidiol y los resultados óseos controlando el sexo, la maduración somática, la masa magra y la estación del año.

Resultados y hallazgos principales

En el **estudio 1**, los niveles de IL-6 y VEGF se asociaron negativamente con el contenido mineral óseo del cuerpo entero, mientras que los niveles de TNF-α (negativamente) e IL-1β (positivamente) se asociaron con el contenido mineral óseo de la columna lumbar. En el **estudio 2**, no se encontró asociación entre los niveles de leptina o adiponectina y los resultados óseos. Además, los altos niveles de fuerza muscular medidos objetivamente pueden atenuar los efectos adversos de VEGF y TNF-α en el contenido mineral óseo del cuerpo entero y en la columna lumbar, respectivamente (**estudio 1**). Además, se observó una asociación negativa entre la leptina y el contenido mineral óseo de la columna lumbar en el grupo de menor fuerza en el tren inferior, mientras que no se encontró una asociación significativa en el grupo de mayor fuerza en el tren inferior (**estudio 2**). En el **estudio 3**, el calcidiol se asoció

indirectamente con la densidad mineral ósea del cuerpo entero, los brazos y las piernas a través de la fuerza muscular relativa.

Conclusiones generales

Los resultados de la presente Tesis sugieren que IL-1β, IL-6, TNF-α, VEGF y leptina (en menor medida) pueden contribuir a explicar el vínculo entre la obesidad y el hueso en niños con sobrepeso u obesidad. Unos niveles óptimos de fuerza muscular pueden atenuar los efectos adversos de TNF-α, VEGF y leptina en los huesos. Además, la fuerza muscular parece jugar un papel importante en la relación de los niveles de calcidiol y el hueso en esta población. En conjunto, los niveles apropiados de vitamina D y de fuerza muscular pueden contribuir a mantener un desarrollo óseo saludable en niños con sobrepeso u obesidad.

3. ABBREVIATIONS

ABBREVIATIONS

$1.25(OH)_2D$: Calcitriol

25(OH)D: Calcidiol

aBMD: Areal bone mineral density

BMI: Body mass index

BMC: Bone mineral content

CRP: C-reactive protein

DXA: Dual-energy x-ray absorptiometry

EGF: Epidermal growth factor

FM: Fat mass

IL: Interleukine

LM: Lean mass

LS: Lumbar spine

NF-κB: Nuclear factor kappa B

PHV: Peak height velocity

RANK: Receptor activator of NF-κB

RANKL: Receptor activator of NF-κB ligand

RM: Repetition maximum

TBLH: Total body less head

TNF-α: Tumour necrosis factor-alpha

VEGF: Vascular endothelial growth factor

4. GLOSSARY

Bone metabolism: Continuous cycle of bone resorption and bone formation carried out by bone cells (i.e., osteoclasts, osteoblasts and osteocytes) in order to maintain bone tissue quantity and quality. In bone diseases like osteoporosis, bone metabolism is altered, leading to bone loss and changes in the microarchitecture that result in bone fragility and ultimately fracture.

Bone modelling: Uncoupled bone resorption-formation process taking place at bone structural units on periosteal, endocortical, peri-trabecular or intra-cortical bone surfaces. The primary function is to increase bone mass and maintaining or altering bone shape. This process usually involves a positive result in young individuals or in those who maintain a reasonable amount of physical activity (modelling is sensitive to mechanical stimulation), and although it decreases with age, never disappears completely.

Bone remodelling: Coupled bone resorption-formation process taking place at bone structural units on periosteal, endocortical, peri-trabecular or intra-cortical bone surfaces (usually observed in periosteal surfaces). The main function of bone remodelling is the replacement of a small amount of the pre-existing bone by a comparable (neutral balance) or a smaller (negative balance) piece of new bone.

Bone turnover: Absolute amount of the whole-body bone mass resulting from bone resorption and formation. A negative turnover balance is the cause of osteopenia and osteoporosis, whereas a positive bone turnover can prevent or improve any kind of osteopenia and osteoporosis. Of note, bone turnover is commonly used to refer bone turnover markers in scientific articles.

Cytokines: Low molecular weight regulatory proteins that are produced by a cell and involved in receptor-mediated cell–cell communication. Cytokines commonly affect cell development and function by autocrine (acting on the same cell that produces the factor) or paracrine (acting on a cell near to that which produces the factor) mechanisms and act at low concentrations (nano- to picomolar).

Growth factors: Molecules that affect cell proliferation, differentiation and wound healing by autocrine and paracrine mechanisms. Thus, a cytokine that induces cell proliferation could be called a growth factor. Nevertheless, we differentiate both terms throughout this Doctoral Thesis.

Inflammation: Coordinated biologic response to the disruption of normal cellular or systemic physiology. Disruptors can be external pathogens (i.e., bacteria, viruses, toxins and infections) or physiologic alterations in response to environmental signals. When the receptors of innate immune cells detect a pathogen-associated molecular pattern, an acute inflammatory response is initiated. In addition, this response can be also activated by damage-associated molecular patterns that are released in response to physical, chemical or metabolic stimuli during cellular stress. Systemic chronic inflammation is commonly activated by this damage-associated molecular patterns.

Muscular fitness: It represents muscular strength, local muscular endurance and muscular power. Briefly, muscular strength is the ability to generate force with a muscle or group of muscles; local muscular endurance is the ability to perform repeated contractions with a muscle or group of muscles under sub-maximal load; and muscular power refers to the rate at which muscles perform work.

Nuclear Factor kappa B (NF-κB): Protein complex that regulate expression of genes involved in numerous normal cellular activities. They are also activated in many inflammatory and neoplastic conditions in which their expression may be stimulated by pro-inflammatory cytokines. NF-κB signalling plays essential roles in certain aspects of osteoclast, osteoblast and chondroblast activities.

Osteoblasts: Responsible cells for bone formation. They originate from mesenchymal stem cells, which also give rise to chondrocytes, muscle cells, and adipocytes (depending on the specific activation of transcription factors). The main function of osteoblasts is carrying out the functions of bone matrix protein secretion and bone mineralization. Osteoblasts express high levels of alkaline phosphatase and osteocalcin, and the circulating concentrations of these proteins reflect the number of osteoblasts and the rate of bone formation.

Osteoclasts: Multinucleated cells involved in bone resorption. They originate from osteoclasts precursors recruited to the bone surface, which are differentiated and fused into mature osteoclasts. The main function of osteoclasts is polarization, acidification and enzymatic dissolution of unnecessary, damaged or old bone tissue. C-terminal crosslinking telopeptide of type 1 collagen is cleaved during osteoclast activity and its blood levels reflect the rate of bone resorption.

Osteocytes: Osteocytes are the most abundant cells in bone. They are former osteoblasts that become entombed during the process of bone deposition and are regularly distributed throughout the mineralized bone matrix. The main function of osteocytes is to coordinate the function of osteoblasts and osteoclasts in response to both mechanical and hormonal cues.

Osteoporosis: Systemic skeletal disease that is characterized by low bone density and microarchitectural deterioration, resulting in decreased trabecular bone number, increased bone fragility and increased fracture risk. Dual-energy x-ray absorptiometry (DXA) is the gold-standard instrument for measuring bone. In this sense, the areal bone mineral density (aBMD) T-score is used to diagnose osteoporosis in postmenopausal women and men older than 50 years, whereas the aBMD Z-score is the outcome used to diagnose osteoporosis in premenopausal women and men younger than 50 years. In children, the diagnosis of osteoporosis can be made in two ways: (1) The finding of one or more vertebral compression fractures, in the absence of local disease or high-energy trauma; (2) the presence of a clinically significant fracture history and aBMD Z-score ≤ -2.0.

Overweight and obesity: Excessive or abnormal fat accumulation that present a risk to health. Commonly, it is determined by body mass index (≥25 kg/m^2 for overweight and ≥30 kg/m^2 for obesity). Body mass index is internationally accepted since it has been reported as a strong predictor of mortality in epidemiological studies and is easy to assess.

RANK: Member of the tumour necrosis factor receptor family. RANK is the receptor for RANK-Ligand (RANKL) and part of the RANK/RANKL/osteoprotegerin signalling pathway that regulates osteoclast differentiation and activation. Osteoprotegerin is a decoy receptor for RANKL and regulates the stimulation of the RANK signalling pathway by competing for RANKL.

RANKL: Member of the tumour necrosis factor family. RANKL is expressed in osteoblasts and muscle tissue among other cells and organs and affects osteoclast differentiation and activation.

5. GENERAL INTRODUCTION

5.1. Childhood obesity: an epidemic affecting bone health

Overweight and obesity are at epidemic levels in developed and developing countries worldwide (1). In fact, this issue has become more common than under-nutrition in adults, with a 39% (39% in males and 40% in females) being overweight or obese (2). In Spain, the mean body mass index (BMI) increased by 0.10% per year in men and 0.26% per year in women from 1987 to 2014, resulting in 34% of adults being overweight or obese (3,4). Importantly, ~23 500 000 Spaniards with overweight/obesity were hospitalised in 2016, which supposed a direct extra-medical cost of 1.95 billion per year (i.e., 2% of the 2016 health burden). Furthermore, if the BMI trend continues, it is expected to increase the number of hospitalisations by 16% and the direct extra-medical costs by 58% in 2030 (3).

Obesity prevention should start at the paediatric stage. Nevertheless, the prevalence in children and adolescents was also increased from 4% in 1975 to over 18% in 2016 (1). In this sense, although Spanish children and adolescents seem to move toward healthier BMIs as they age (5), gaining excess weight during the paediatric stage is likely to lead to overweight/obesity into adulthood (6). Childhood obesity is linked with psychological (e.g., low self-esteem) and cardiovascular problems (i.e., high blood pressure, dyslipidaemia, abnormalities in left ventricular mass and/or function, abnormalities in endothelial function and insulin resistance) in the short term (7). Furthermore, in the long term, childhood obesity markedly increases susceptibility to a range of associated chronic diseases (i.e., cardiovascular diseases, type 2 diabetes and cancer) (8) and physical limitations (i.e., musculoskeletal disorders) (9–11), which lead to disability pension in the future (12), as well as

morbidity and mortality (13). Otherwise, the relationship with bone health has been extensively investigated, but the results are inconclusive (14).

Bone health is defined as a public health issue with an emphasis on prevention and early intervention to promote strong bones and prevent fractures and their consequences (15). In this regard, acquiring an optimal peak bone mass during the growth stage is a suitable measure to preserve bone health across the lifespan, and could delay the onset of osteoporosis (16,17). Peak bone mass has a strong genetic component, although lifestyle factors (i.e., physical activity and dietary habits) contribute up to 20% of the variation (18). In general, children and adolescents with overweight/obesity usually have a greater bone mineral content (BMC) and areal bone mineral density (aBMD) than their normal-weight peers as they mature earlier, tend to be taller and have greater lean mass (19–21). However, a longitudinal study reported that boys with overweight/obesity who had an extensive BMI gain during puberty experienced lower gains in bone outcomes (22). Additionally, the increased rate of fractures in this population suggests a poorer bone quality (23). The latter results are supported by the following facts: i) excessive fat mass accumulation interacts with bone by secreting various inflammatory cytokines and adipokines (24); ii) bone marrow adipose tissue affects aBMD and bone microstructure (25); and iii) nutrient consumption imbalances such as vitamin D, calcium and phosphorus, which are known to affect bone metabolism (26).

Therefore, in the present International Doctoral Thesis, the target population are children with overweight/obesity in which the role of systemic chronic inflammation, vitamin D and muscular fitness in bone health needs to be elucidated.

5.2 Obesity-associated inflammation in children

Despite the intermittent increases in inflammation are crucial for human survival during infection or physical injury, scientific evidence has stated that environmental, social and lifestyle factors may induce systemic chronic inflammation (27). This systemic-chronic-inflammatory state often increases with age as reported by studies showing higher levels of circulating cytokines, chemokines and acute phase proteins in older individuals, and this is thought to be caused by a process called cellular senescence (28). In addition to its tumour-suppressive mechanism, the senescence seems to develop a multifaceted senescence-associated phenotype that turns senescent fibroblasts into proinflammatory cells which contributes to the inflammatory status (29). However, the origin of systemic chronic inflammation can be understood from a developmental perspective since childhood environment impacts metabolic and immune responses later in life (30).

Childhood obesity involves an excessive fat accumulation, in particular, in the visceral depots which causes systemic chronic inflammation (31,32). When visceral adipose tissue is expanded, an hypoxia micro-environment emerges which induces necrotic cell death of adypocites (33). Necrosis results in the release of damage-associated molecular patterns that macrophages recognise and thus produce pro-inflammatory cytokines (34). Consequently, the inflammatory profile seems to be different between obese and lean children (35–39). In this sense, higher serum interleukine (IL)-1β, IL-6, tumour necrosis factor-alpha (TNF-α), epidermal growth factor (EGF), c-reactive protein (CRP) and leptin levels were found in children with obesity compared to lean children, whereas no difference in vascular endothelial growth factor (VEGF) was observed (35,36,38,39). Furthermore, lower levels of

serum adiponectin have been observed in children and adolescents with obesity (37). However, two studies found no difference in the concentration of IL-6, TNF-α and adiponectin between obese or non-obese adolescents (39,40). Interestingly, Kleiner et al. (41). have found that IL-6 and TNF-α were upregulated in healthy children and adolescents between 7 and 17 years, whilst IL-β were under the lower limit of detection and VEGF levels remained constant from childhood to adulthood. This Doctoral Thesis is focused on the aforementioned inflammatory markers and adipokines: IL-1β, IL-6, TNF-α, CRP, EGF, VEGF, leptin and adiponectin. Hence, a short description of the biochemical markers and their role in the human body is given (**Table 1**).

Table 1. Summary of the biochemical markers description

Biochemical marker	Source	Functions/effects	Childhood-obesity associated
IL-1β	Monocytes	Mediator role in innate immunity (by TNF-α production) ↑ Insulin resistance ↓ Adiponectin	Controversial
IL-6	Macrophages Fibroblasts endothelial cells Muscle tissue Adipose tissue	Mediator role in innate immunity (by IL-1β production) ↑ Lipolytic effect ↑ Glucose homeostasis ↑ Insulin resistance ↑ SCI	Yes
TNF-α	Macrophages Lymphocytes Adipose tissue (less extent)	Mediator role in innate immunity (by leptin production) ↑ Apoptosis ↑ Insulin resistance ↑ Dyslipidemia ↓ Endothelial function ↓ Adiponectin	Controversial
CRP	Liver	Mediator role in innate immunity (by IL-6 production) ↑ Phagocitosis ↑ Leptin	Yes
EGF	Salivary gland	Proliferation, differentation and migration of epithelial cells	Yes
VEGF	VEGFA and Angpt2 genes (in response to hypoxia)	Angiogenesis ↑ MSC differentiation into adipocytes ↑ Insulin resistance	Yes
Leptin	Adipose tissue	Mediator role in innate-adaptative immunity Appetite regulation Pubertal development Angiogenesis Hematopoiesis Bone metabolism ↑ Leptin resistance ↑ Insulin resistance	Yes
Adiponectin	Adipose tissue	Anti-inflammatory role ↓ SCI ↓ Atherosclerosis ↓ Insulin resistance	Yes (inversely)

IL interleukin, *TNF-α* tumour necrosis factor-α, *CRP* c-reactive protein *EGF* epidermal growth factor, *VEGF* vascular endothelial growth factor A, *SCI* systemic chronic inflammation, *MSC* mesenchymal stem cell.
Functions/effects in black colour are due to the acute inflammatory response
Functions/effects in blue colour are due to the normal concentrations of the biochemical markers
Functions/effects in red colour are due to the chronic elevated concentrations of biochemical markers

IL-1β belongs to IL-1 family of cytokines and receptors which are key mediators of innate inflammatory response. IL-1β is mainly produced by monocytes in response to infection, injury or immunologic challenge; it causes fever, hypotension and production of other pro-inflammatory cytokines, such as IL-6 (42). Together with IL-1α, IL-1β is responsible for several metabolic effects (43). In this regard, IL-1α may play a role in the development of obesity, whilst IL-1β may play a role in the development of obesity associated with insulin resistance (44,45). Although cytokines of the IL-1 family have been described to be elevated in adults with overweight (46), results from studies with adolescents are contradictory. For instance, IL-1β concentration has been shown to be higher in children and adolescents with obesity, and positively associated with BMI (38,47). However, Jung et al. (48) found that IL-1β levels were below the detection threshold in both obese and lean children.

IL-6 is a cytokine with various functions such as host defense, bone metabolism and tissue injury (42,49). IL-6 is produced by many cell types and tissues, including immune cells, fibroblasts, endothelial cells, skeletal muscle and adipose tissue (49) and appears to have both pro- and anti-inflammatory activity depending on the tissue and metabolic state (50). Adipose cells contribute 15 to 30% of circulating IL-6 in the absence of acute inflammation (51) and correlation between serum IL-6 and the level of obesity has been shown (52). IL-6 has a pivotal role in metabolic processes having lipolytic effects and anti-obesity potential (53,54). On the other hand, the transient increase in IL-6 may assist in normal glucose homeostasis, whereas the relentless increase in systemic level of IL-6 may lead to insulin resistance (50). Interestingly, IL-6 levels in central nervous system are negatively correlated with FM in humans with overweight, suggesting central IL-6 deficiency in obesity (55). With regard to

paediatric population, Herder et al. (56) showed that serum IL-6 was associated with BMI, waist circumference and insulin resistance in adolescents, whilst Martos-Moreno et al. (57) reported that IL-6 levels decrease during pubertal development in both sexes.

TNF-α is produced mainly by macrophages and lymphocytes, and to a less extent also by adipose tissue (58). TNF-α plays a central role in inflammation, immune system development, apoptosis and has numerous effects in adipose tissue, including lipid metabolism and insulin signalling (42,49). Petersen and Pedersen (54) suggested that TNF-α rather than IL-6 is the driver behind insulin resistance and dyslipidemia. Notwithstanding this, Aycan et al. (59) found similar concentration of TNF-α in hyperinsulinemic and normoinsulinemic children with obesity. TNF-α has also an effect on endothelial function in children with obesity (49). Although higher levels of TNF-α have been found in children and adolescents with obesity (47), Herder et al. (56) did not find association between serum TNF-α concentration and BMI or waist circumference.

CRP is an acute-phase protein synthesized by the liver in response to factors released by macrohages and adipocytes (e.g., IL-6) (60), and it was the first pattern recognition receptor to be discovered (61). CRP binds to the phosphocholine expressed on the surface of dead (or dying cells) and some bacteria. Then, complement system is activated, promoting phagocitosis by macrophages that clears necrotic and apoptotic cells and bacteria (62). Obesity has been associated with elevated levels of CRP in both adults and children (63). Moreover, Juonala et al. (64) reported that elevated levels of CRP in childhood and adolescence may track into adulthood, which can also influence leptin levels (65).

EGF is a member of growth factors and plays important role in proliferation, differentiation and migration of a variety of cells, especially in epithelial cells (66). In the adult organism, EGF plays essential roles in the proliferation and differentiation of the mammary gland at puberty and mammary gland milk production during pregnancy (67). EGF receptors are highly expressed also in adipose tissue (68). Inverse associations of serum EGF level with fat mass and BMI have been found in adults (69,70). However, a positive correlation was found between EGF and increased BMI in children and adolescents (35).

VEGFs is a group of growth factors involved in angiogenesis, vascular permeability, lymphangiogenesis and neuronal development (i.e., VEGFs A-E, placental growth factor and the snake venom VEGF-F) (71,72). By convention, VEGF is referred to VEGFA and it is highly expressed in adipose tissue (73,74). Adipose VEGF is critical for maintaining the viability and metabolic/endocrine function of adipocytes through its role in regulating adequate vascularization and blood perfusion (73). A positive correlation between serum VEGF concentration and BMI has been shown in adults (75,76). High levels of VEGF have been shown to coincide with a trend towards lower insulin sensitivity in children and adolescents with obesity (35).

Leptin is a hormone mainly produced by adipose tissue (49). Effects of leptin are associated mainly with appetite regulation through neuropeptide Y and energy metabolism, but also with pubertal development, reproduction, immune system, hematopoiesis, angiogenesis, bone formation and wound healing (49,77). Leptin concentration is strongly and positively correlated with BMI and fat mass (77,78). Leptin rises similarly over the pre-pubertal years into early puberty in both sexes,

thereafter declines to nadir in boys at pubertal stage 5, but keeps rising until a peak in girls at pubertal stage 5 (77).

Adiponectin is an adipose-secreted hormone that has been shown to exert anti-inflammatory effects on macrophages (79). Adiponectin could protect against chronic inflammation, insulin resistance, atherosclerosis and cardiovascular diseases (80). Adiponectin levels decline with age and progression of puberty (37,81). Adiponectin has been shown to correlate negatively with BMI in children and adolescents (82,83), and its levels increase in conjunction with body mass loss (49). Furthermore, central obesity has been shown to lower adiponectin levels through increasing pro-inflammatory cytokines such as TNF-α, IL-1β and leptin (49,84).

5.3. Inflammatory markers and adipokines as biological mechanisms responsible for bone health: The bone-adiposity crosstalk

The scientific evidence has highlighted the possibility that bone metabolism may be integrated in the adipose tissue network (85,86). In this context, three possible mechanisms may underlie the effects of adiposity on bone turnover: i) cytokines and growth factors released by adipocytes affect osteoblasts and osteoclasts; ii) adipokines (e.g., leptin and adiponectin) regulate central nervous system outflow from the sympathetic nervous system; and iii) paracrine factors secreted by adipocytes within the bone marrow milieu influence nearby cells on the trabecular bone surface (87,88).

Several studies have reported that the paediatric skeleton is affected by inflammatory markers such as IL-1β, IL-6, TNF-α, CRP, EGF and VEGF (89–91). Likewise, Dimitri et al. (92) found that leptin negatively correlates with different bone

parameters in children with obesity, whereas no correlation was found with adiponectin. Altogether, this Doctoral Thesis aimed at examining the association between the previously described inflammatory markers and adipokines, and bone outcomes. Therefore, a background on the effect of these biochemical markers on bone outcomes is provided (**Table 2**).

Table 2. Summary of the biochemical markers' effects on bone metabolism

	Osteoblast/Osteocyte			Osteoclast	
Biochemical marker	Differentiation	Function (bone formation)	Proliferation or survival	Differentiation	Function (bone resorption)
IL-1β	↑	↓	↓	↑ (via ↑RANKL and ↓OPG)	↑
IL-6	↓ (by interacting with ALP activity)	Unknown	↓	↑ (via ↑RANKL and ↓OPG)	Unknown
TNF-α	↓	↓	↓	↑ (via NF- κB, JNK and p38)	↑ (via NFATc1)
CRP	↓	↓	↓	Unknown	↑ (via ↑RANKL)
EGF	↑	Unknown	↑	↑	Unknown
VEGF	↓	Unknown	Unknown	↑	↑
Leptin	↑, ↓	↑	↑	Unknown	↑ (via ↑RANKL)
Adiponectin	↑	Unknown	Unknown	↓	Unknown

IL interleukin, *TNF-α* tumour necrosis factor-α, *CRP* c-reactive protein, *EGF* epidermal growth factor, *VEGF* vascular endothelia alkaline phosphatase, *NF-κB* nuclear factor kappa B, *RANK* receptor activator of NF-κB, *RANKL* receptor activator o osteoprotegerin, *NFATc1* nuclear factor of activated T cells. Adapted from Plotkin et al. (93)

IL-1β is a proinflammatory marker associated with osteoclastogenesis via induction of receptor activator of nuclear factor κ-B ligand (RANKL) and inhibition of osteoprotegerin, and also reduces osteoblast recruitment in vitro (94). In this regard, IL-1β has been negatively associated with lumbar spine (LS) and femoral neck aBMD in older adults (95) and negatively associated with hip and LS aBMD in postmenopausal women (96). However, a longitudinal study found no associations between increments in IL-1β levels and bone parameters in overweight pubertal boys (90).

High levels of IL-6 and IL-6 receptor (IL-6R) have been documented to affect osteoblast differentiation since IL-6 signalling strongly interferes with alkaline phosphatase activity, which downregulates the expression of osteoblastic genes (i.e., RUNX2, Osterix and osteocalcin) and reduces the rate of mineralization (97). Three signalling pathways are activated when IL-6R binds to extracelullar IL-6: SHP2/MEK/ERK, SHP2/PI3K/AKT2 and JAK/STAT3. The first two signalling pathways downregulate osteoblastogenesis, but the JAK/STAT3 signalling pathway acts both as a negative and positive regulator of osteoblast differentiation, leading to a net effect of decreased osteoblastogenesis (98). Previous studies have reported that elevated IL-6 levels were negatively associated with hip aBMD and LS aBMD in adults (99). Furthermore, Hanks et al. (89) found a negative correlation between IL-6 and BMC in prepubertal girls. Likewise, Mengel et al. (90) found a negative correlation between increments in IL-6 concentrations and LS aBMD in pubertal boys.

TNF-α is also an inhibitor of osteoblast differentiation and an activator of osteoclastogenesis (100). Directly, TNF-α binds to TNF receptor-1 and activates NF-kB, JNK and p38 pathways, which promotes the transcription of genes involved in

osteoclast formation and activity. Moreover, TNF-α indirectly affects osteoclast formation by promoting RANKL expression in bone marrow stromal cells (101). Few studies have documented this role in humans (90,95,102). Zheng et al. (95) reported that TNF-α produced by stimulated whole blood cells was inversely associated with LS aBMD in postmenopausal women, whereas Ding et al. (102) found that the inverse association between serum TNF-α and LS aBMD in older men disappeared after controlling for IL-6. Likewise, Mengel et al. (90) showed no association between increments in TNF-α and aBMD in overweight pubertal boys.

CRP has been documented to affect bone by binding to extracellular leptin, which impairs the leptin functions on bone metabolism (65). The association between CRP and aBMD or fracture risk has been at the scope of several studies (103–105). Findings from the Tromso study showed an inverse association between high-sensitivity CRP and aBMD in men after adjusting for BMI. Additionally, high sensitivity CRP was associated with increased fracture risk in both sexes (106). Indeed, Lucas et al. (107) demonstrated that higher levels high-sensitivity CRP were associated with decreased aBMD in adolescent girls with overweight.

EGF seems to stimulate bone resorption by increasing the proliferation of osteoclast precursors, which leads to increased numbers of osteoclasts (108). Specifically, EGF-like ligands regulate the expression of two secreted osteoclast regulatory factors in osteoblasts by decreasing osteoprotegerin expression and increasing monocyte chemoattractant-1 expression and consequently stimulate tartrate-resistant acid phosphatase that leads to osteoclast formation (109). In fact, Mengel et al. (90) demonstrated that increments in EGF levels were negatively associated with LS aBMD in pubertal boys with overweight. On the other hand, when

EGF binds to its receptor proliferation and survival of osteoblasts may be also occurring by increasing early growth response-2 expression (110).

VEGF is known to directly induce osteoblast and osteoclast differentiation and therefore bone formation and remodelling (111). In mesenchymal stem cells, intracellular VEGF controls the balance between osteoblast and adipocyte differentiation. Likewise, VEGF controls osteoclast differentiation, migration, and activity by upregulating the expression of receptor activator of NF-κB (RANK) and RANKL and by stimulating PI3K/Akt signalling (autocrine pathway) (93). Furthermore, a novel role for osteocytes as a source of VEGF has emerged. Thereby, osteocytes located in the vicinity of areas exposed to damage produce increased levels of VEGF in response to mechanical loading, which may be required to promote angiogenesis and osteoclastogenesis, primary steps in bone remodelling (93).

Leptin exerts both central and peripheral actions on bone (112). In the central pathway, leptin binds to its receptor in the ventromedial hypothalamus and induces an increase in sympathetic activity that signals to osteoblasts via the b2 adrenergic receptors (113). Thereby, leptin suppresses osteoblast proliferation and/or increases the expression of the receptor of RANKL activator which promotes the resorption of osteoclasts (114). In the peripheral pathway, leptin enhances the proliferation and differentiation of mesenchymal stem cells into osteoblastic lineage (115). The overall effect appears to be beneficial for bone formation in mice (116). Otherwise, leptin has been proposed to inhibit bone turnover in obese humans (117–119).

The role of the adiponectin on bone metabolism is inconclusive. A recent review indicated that adiponectin acts to promote osteoblast differentiation within the bone marrow niche and simultaneously inhibiting osteoclastogenesis, although these

mechanisms might be dysregulated in obese individuals (120). Importantly, weight loss in adolescents with obesity was associated with increased adiponectin levels coupled to lower levels of the bone resorption marker, collagen β c-terminal telopeptide (121).

5.4. Mechanical bone adaptation: *'May the forcer be with you'*

Although it appears dead and unresponsive, bone is one of the most adaptable tissues and organs in the human body. This was recognized nearly 200 years ago, when surgeons noticed that trabecular bone in the femoral head and neck was oriented to diaphysis in order to redirect the stress to the stronger cortical shell (**Figure 1**) (122).

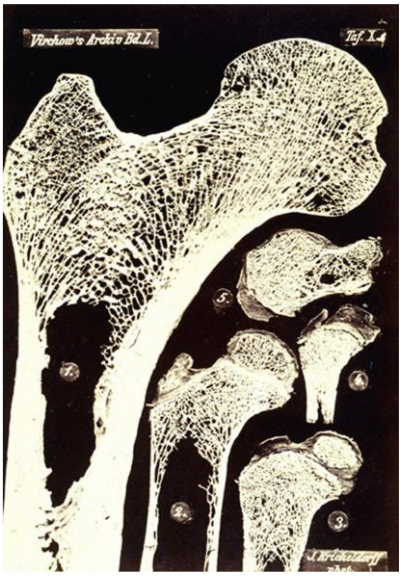

Figure 1. Trabecular alignment in a frontal longitudinal section of the proximal femur. (i) A frontal longitudinal section of the proximal femur from a 31-year-old man. (ii) A frontal longitudinal section from the proximal femur of a 3-year-old girl, (iii) a 1-year-old boy, (iv) a new-born and (v) a sagittal section through the calcaneus of a 5-year-old girl. With permission from Wolff et al. (123)

Bone receives stress (external force, Pascals [Pa]) which produces strain (structural deformation, microstrains [με]). According to Wolff's law, bone remodelling is stimulated by mechanical strain as a result of applied mechanical stress

in the form of muscular contraction, impact loading and gravitational forces (124,125). Likewise, the Mechanostat Theory describes the relationship between strains and subsequent adaptations of bone mass and geometry (**Figure 2**) (126). This theory suggests that bone cells monitor how the bone is stressed, integrate this information, and stimulate bone remodelling to either increase or decrease bone mass in response to specific strain magnitude and modes (126–128). Thus, when the strain magnitude sits below the minimum effective strain threshold (i.e., <800 με), bone resorption occurs to eliminate unnecessary bone mass. Otherwise, when the strain magnitude exceeds the minimum effective strain threshold (>1500 με), bone formation occurs to increase bone mass and cross-sectional area (127,129).

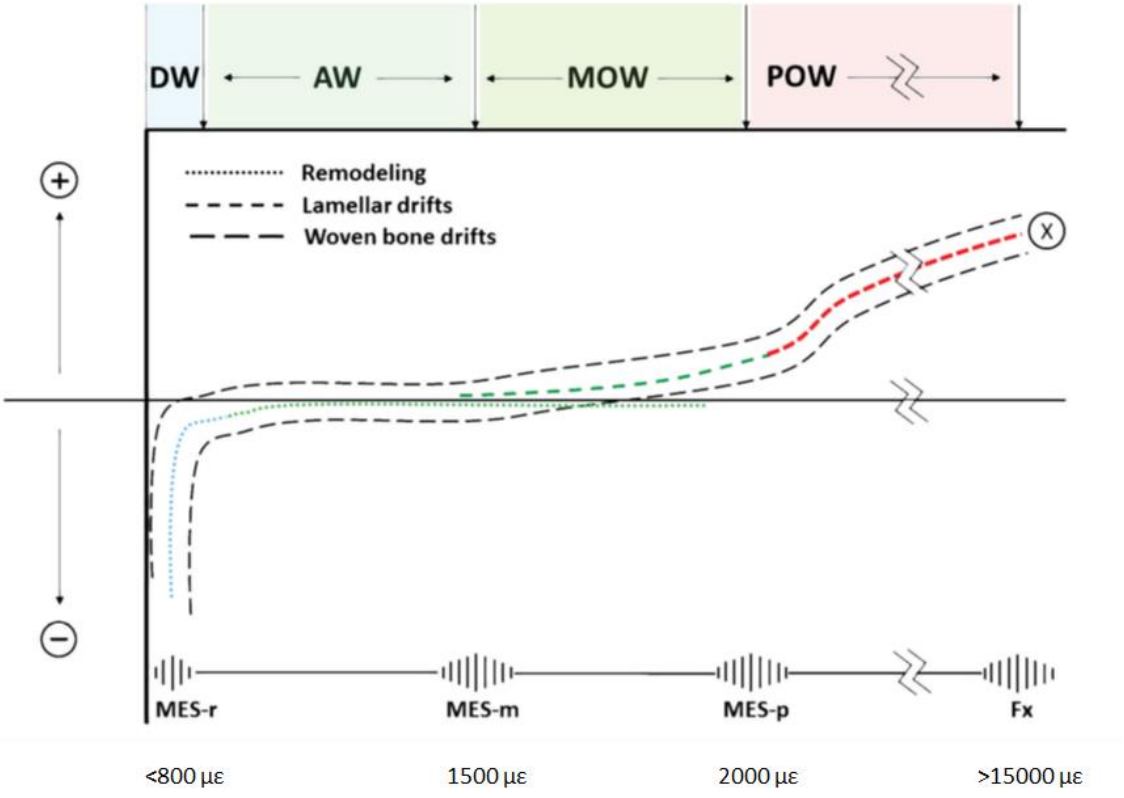

Figure 2. Mechanostat Theory: Modelling and remodelling effects on bone strength and mass. DW= disuse window; AW= adapted window; MOW = mild overload window; POW= pathologic overload window; MES= minimum effective strain (r= remodelling, m= modelling, p= microdamage), Fx= fracture strain. With permission from Hart et al. (128)

Notwithstanding the strain magnitude, bone response is also affected by strain frequency, distribution, number of loading cycles and rest-recovery periods (125,130,131). Altogether, the American College of Sports Medicine stated a guideline on physical activity for bone health (**Table 3**) (132).

Table 3. Guidelines for physical activity prescription aimed at improving bone health

FITT criteria	Children and Adolescents	Adults
Frequency	At least 3 days/week	3-5 days/week of weight-bearing endurance activities 2-3 days/week of resistance exercise
Intensity	High, in terms of bone-loading forces. <60% of 1RM in case of resistance training	Moderate to high, in terms of bone-loading forces
Time	10-20 min (2 times per day or more may be more effective)	30–60 min of a combination of weight-bearing endurance activities, activities that involve jumping, and resistance exercise that targets all major muscle groups
Type	Impact activities, such as gymnastics, plyometrics, and jumping, and moderate intensity resistance training; participation in sports that involve running and jumping (133,134)	Weight-bearing endurance activities (tennis; stair climbing; jogging, at least intermittently during walking), activities that involve jumping (volleyball, basketball), and resistance exercise (weight lifting)

With regards to children and adolescents with obesity, a meta-analysis showed that physical activity interventions did not influence BMC and aBMD, but had positive effects on lean and fat mass (21). Of note, half of the studies included in this meta-analysis involved physical activity and nutritional interventions (aimed at weight loss), the descriptions of physical activity interventions were not well described, and they were short term. In this sense, Kondiboyina et al. (135) recently showed that a 9-month exercise programme based on aerobic and resistance activities at moderate to vigorous intensity improved aBMD in children with overweight/obesity. Collectively, high-magnitude and low-frequency strains (e.g., vigorous physical

activity and plyometric exercise) seems to be the best choice to improve bone mass in different populations (134,136–139).

Thereby, the muscle forces acting on bone must be rapid, powerful, and changing in magnitude and direction in order to stimulate bone adaptation (130). Of note, the morphology of connective tissue may play a role (140,141). In this context, Harold Frost combined the bone and muscle physiology with the Mechanostat Theory to develop the Utah paradigm of skeletal physiology (142). This paradigm states that larger muscles exert greater forces on the bones, which will adapt and therefore improve their strength. Moreover, previous studies have reported a mediator role of lean mass in the association between muscular fitness and bone mass in children (143,144). A meta-analysis showed that optimal muscular fitness levels during childhood and adolescence are paramount for future bone health (145). On the basis of the above, Torres-Costoso et al. (146) have found that both fat and unfat individuals with higher levels of muscular fitness showed better BMC and aBMD, suggesting that the 'fat but fit' paradox also occurs in the context of bone health.

The present Doctoral Thesis provides, for the first time, an observational perspective of the moderator role of muscular fitness in the association between either inflammatory markers or adipokines and bone health in children with overweight/obesity. To this end, multiple regression analyses with interaction effect allow us to understand when the independent variable is associated with the dependent according to a third variable (i.e., the moderator). Moreover, PROCESS macro is used to specify the moderator's cut-off point above/below which the direction and/or significance of the association is modified (147).

5.5. Vitamin D implications for bone health in growing children

Vitamin D is an essential molecule for human physiology. To date, there exist two major forms of vitamin D with different chain structure, but same function. Vitamin D_3 (or cholecalciferol) which is synthesized in the skin of humans in response to sun exposure and consumed in the diet (148). On the other hand, vitamin D_3 (or ergocalciferol) is derived from plant sources and it is not human-made or added to foods (149). After their absorption or photobiogenesis, they can be storage in human tissues (mainly in adipose tissue), or hydroxylated by the liver which form calcidiol (25[OH]D) and released into blood (150). Of note, this 25(OH)D levels have 3 weeks of life on average. When the levels of calcium and phosphorous are low, 25(OH)D is hydroxylated in the kidney into calcitriol ($1.25[OH]_2D$), which regulates different physiological processes in human body due to the vitamin D receptor (VDR) is present in many tissues of the human body (151,152). The most widely known function of vitamin D is the regulation of calcium and phosphorus homeostasis and bone calcium mobilisation (153).

Vitamin D is considered an important determinant of bone health at school age (154,155). Sex, age, race, season of the year in which blood was collected and sun exposure have been associated with levels of 25(OH)D (156). Moreover, obesity has been associated with 25(OH)D deficiency independent of age, latitude and the cut-offs to define deficiency (157). Childhood obesity is associated with a deficient-25(OH)D status in Spain (158). In this context, there are two components that we must take into account in this population: Vitamin D sequestration by adipose tissue and decreased exposure to sunlight as a consequence of the sedentary lifestyle (159,160). Several studies have shown that 25(OH)D-deficient children had lower LS

aBMD and TB aBMD Z-score (154,161). These findings are thought to be related to parathormone levels and calcium metabolism. Nevertheless, as VDR is present in the skeletal muscles, we hypothesize that low levels of 25(OH)D may affect muscular fitness, and thus the muscle-bone unit.

5.6. Role of vitamin D in the muscular fitness: A novel perspective in the study of bone in relation to vitamin D

As previously stated, having optimal muscular fitness levels at young age are crucial for bone health later in life (145). Furthermore, the relationship between muscular fitness and bone health is known to be mediated by muscle mass (143,144). The contraction of the muscle mass is the physiological mechanism for muscle function, which has been reported to be influenced by vitamin D (162). In this regard, 1.25$(OH)_2$D binds to the VDR in the plasma membrane and nucleus of the muscle cells which affect Ca^{2+} handling and muscle cell proliferation and differentiation (163,164). Thus, it is not surprising that sufficient-25(OH)D status is related to higher muscular fitness, enabling prevention of falls in old population (165). Also, vitamin D supplementation with 800-1000 IU per day may reduce the risk of falling through improving muscular fitness and balance in this population (166).

Less is known about this relationship in growing population. A higher upper-body muscular fitness was found in Chinese adolescent girls with sufficient-25(OH)D status compared with those with deficient or severely deficient levels (167). Similarly, Ward et al. (168) observed a positive association between 25(OH)D levels and lower-body muscular fitness in British adolescent girls. Moreover, a recent study showed that higher levels of 25(OH)D were associated with higher relative upper-body muscular

fitness in a nationally representative sample of US youth (169). Despite the relationship between vitamin D, muscular fitness and bone health has been described, no studies have jointly examined the associations of these predictors with bone outcomes. Consequently, in the present Thesis we aim to disentangle whether the relationship between vitamin D and bone health is mediated by muscular fitness in a sample of children with overweight/obesity. For this purpose, simple mediation analyses with PROCESS macro were performed (147). This analysis allows us to understand why the independent variable is associated with the dependent through a third variable (i.e., the mediator). Briefly, this analysis is carried out under the basis that the independent variable (calcidiol) causes the mediator (muscular fitness), and the mediator causes the dependent variable (bone health).

6. AIMS

Overall, the aim of the present International Doctoral Thesis was twofold: i) to study the association of inflammatory markers and adipokines with bone health in children with overweight/obesity, and if so, whether muscular fitness modifies these associations; and ii) to examine the mediator role of muscular fitness in the association between vitamin D and bone health. This overall aim is addressed in two sections:

Section 1: Moderator role of muscular fitness in the association of inflammatory markers and adipokines with bone health.

- To examine which inflammatory markers are associated with bone mass and whether this association varies according to muscular fitness in children with overweight/obesity (**Study 1**).
- To investigate the relationships of adipokines with bone outcomes and whether these relationships are moderated by muscular fitness (**Study 2**).

Section 2: Mediator role of muscular fitness in the association between vitamin D and bone health.

- To examine whether the relationship between 25(OH)D and aBMD is mediated by muscular fitness in children with overweight/obesity (**Study 3**).

7. OVERALL METHODS

The present International Doctoral Thesis was carried out under the umbrella of ActiveBrains project. This project is a randomised controlled trial aimed at improving brain health, as well as physical and mental health, through a 20-weeks exercise programme in prepubertal children with overweight/obesity. However, for the purpose of this Thesis all studies included are based on the ActiveBrains baseline data (cross-sectional design).

Table 4. Inclusion and exclusion criteria of the ActiveBrains project

Inclusion criteria	Exclusion criteria
• To be overweight or obese based on the World Obesity Federation (formerly named International Obesity Task Force) cut-off points. • To be 8 to 11 years old. • Not to have any physical disabilities or neurological disorder that affects their physical performance. • In the case of girls, not to have started the menstruation.	• To be left-handedness. • To be diagnosed with Attention-Deficit Hyperactivity Disorder. • To be diagnosed with other psychiatric disorders.

The present Doctoral Thesis is focused on bone health. Despite the sample size and power calculations were performed based on brain health variables, the sample size included in the three studies was enough as previously published studies on bone health have shown (90,170). **Table 5** shows an overview of the design, participants and variables included in each study.

Table 5. Study methodology overview

	Specific aim	Participants	Predictors	Moderators/ mediators	Bone outcc
Study 1	To examine which inflammatory markers are associated with bone mass and whether this association varies according to muscular fitness in children with overweight/obesity.	55 children with overweight/obesity (10.2 ± 1.2, 69% boys)	IL-1β IL-6 TNF-α CRP EGF VEGF	Muscular fitness	TBLH LS BM
Study 2	To investigate the relationships of adipokines with bone outcomes and whether these relationships are moderated by muscular fitness	84 children with overweight/obesity (10.0 ± 1.2, 63 boys)	Leptin Adiponectin	Muscular fitness	TBLH TBLH aBMD LS BM LS aB
Study 3	To examine whether the relationship between 25(OH)D and aBMD is mediated by muscular fitness in children with overweight/obesity.	81 children with overweight/obesity (10.0 ± 1.2, 65% boys)	25(OH)D	Muscular fitness	TBLH aBMD Arms Legs

IL interleukin, *TNF-α* tumour necrosis factor-α, *CRP* c-reactive protein, *EGF* epidermal growth factor, *VEGF* vascular
TBLH total body-less head, *LS* lumbar spine, *BMC* bone mineral content, *aBMD* areal bone mineral density, *25(OH)D*

The main statistical analyses performed in this Thesis are shown in **Figure 3**. First, multiple linear regression with interaction effects were carried out for study 1 and 2. This analysis allowed us to examine whether the associations of inflammatory markers and adipokines (predictor) on bone parameters (outcome) are modulated by muscular fitness (moderator) (**Figure 3A**). The statistical equation for this analysis is as follow: $Y=b_0 + b_1X_1 + b_2X_2 + b_3X_1X_2$. Where $b_3X_1X_2$ (and its significance) represents the interaction between the predictor and the moderator on the outcome. Moreover, the PROCESS macro by Hayes et al. (147) allowed us to implement a bias-corrected bootstrap method in order to provide greater resolution for clarifying the interaction in the study 2. Specifically, this method uses the Johnson-Neyman technique that seeks for specific muscular fitness cut points in which the relationship between adipokines and bone outcomes changes (i.e., direction and/or significance).

Second, simple mediation analyses were performed to test whether the association between 25(OH)D (predictor) and bone parameters (outcome) was mediated by muscular fitness (mediator). For this purpose, the PROCESS macro was also used under the assumption of two steps (171): (i) that the causal variable is correlated with the mediator (path a); (ii) show that the mediator affects the outcome variable controlling for the predictor (path b). Thus, mediation is assessed by the indirect effect of the 25(OH)D (predictor) on aBMD (outcome) through muscular fitness (mediator). Indirect effects with confidence intervals not including zero were interpreted as statistically significant regardless of the significance of the total effect (path c) and the direct effect (path c', the effect on the outcome when both predictor and mediator are included as independent variables) (**Figure 3B**).

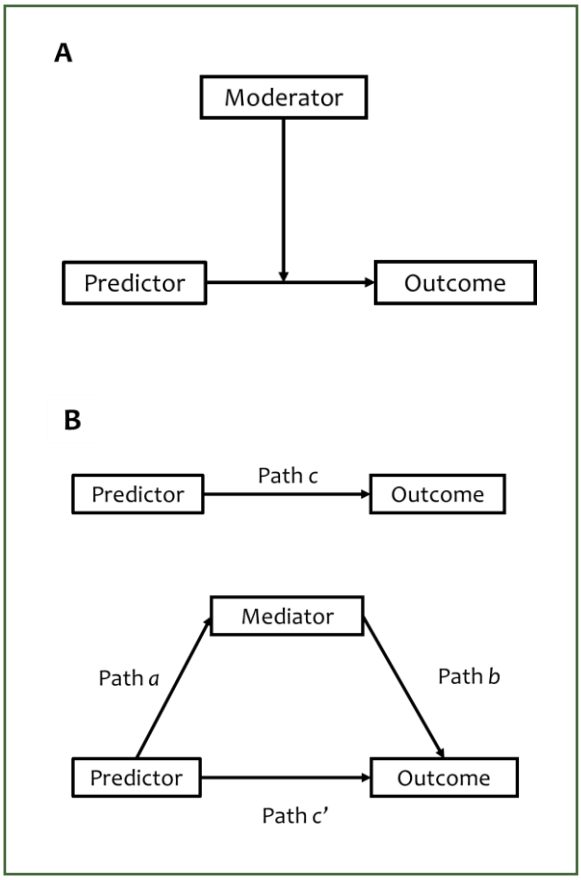

Figure 3. Causal diagrams reflecting the simple moderation (A) and mediation (B) analyses. Path c shows the association between the predictor and the outcome. Arrows a x b show the natural indirect effect pathway, and c shows the natural direct effect pathway. aBMD: areal bone mineral density

8. STUDIES' METHODS, RESULTS AND DISCUSSION

Section 1

Moderator role of muscular fitness in the association of inflammatory markers and adipokines with bone health.

Study 1

Inflammatory markers and bone mass in children with overweight/obesity: The role of muscular fitness.

Introduction

Obesity and osteoporosis are two major global health problems with an increasing prevalence and closely related to both mortality and morbidity worldwide (172,173). The belief that obesity is protective against osteoporosis has recently come into question due to the increasing evidence about the endocrine function and interplay between different tissues, such as muscular, adipose, and bone tissue (174). Moreover, childhood is a critical period for bone accretion (18) and reaching an optimal peak bone mass is considered the best protective factor against future osteoporosis and fracture.

The link between body composition and bone health in children and adolescents has been the focus of various investigations over the last few decades (175). Previous cross-sectional evidence highlights a negative association between fat mass (FM) and bone mass in adolescents, once lean mass (LM) is accounted for (176). In addition, Mengel et al. (22) observed that boys with overweight/obesity who had an extensive body mass index (BMI) gain during puberty experienced lower gains in bone outcomes. In this regard, the increasing presence of fat within the bone marrow is known to affect osteoblast differentiation, increasing osteoclastic activity and affecting mineralization (177).

The pediatric skeleton is sensitive to factors that influence bone accrual, including physical activity and increased inflammatory cytokines (178). In this sense, the role that inflammatory markers play in the child's skeleton has been investigated (89–91). For instance, Mengel et al. (90) found that vascular endothelial growth factor was inversely associated with total body bone mineral content (BMC), whereas epidermal growth factor (EGF) was inversely associated with areal bone

mineral density (aBMD) and apparent bone mineral density at the lumbar spine (LS). Similar to adipose tissue, the skeletal muscle is a secretory organ responsible for the production of several hundreds of myokines in response to exercise (179). Thus, the muscle-adipose tissue axis should be taken into consideration to elucidate the systemic effects of the inflammatory markers on bone health.

Muscular fitness has been favorably associated with potential health benefits (i.e. bone health, mental health, total and central adiposity, cardiovascular disease and metabolic risk factors) in children and adolescents (180). Recent studies reported that the association between muscular fitness and bone outcomes was explained by LM in different growth stages (144,181,182). In addition, muscular fitness has been inversely associated with c-reactive protein (CRP) in adolescents with overweight/obesity (183,184) and also in prepubertal children (185). In adolescents, a clustered score of inflammatory markers including CRP, C3, C4, fibrinogen, and leptin has been inversely associated with muscular fitness (186).

To the best of our knowledge, no study has tested the role of muscular fitness in the association between inflammatory markers and bone mass. Therefore, the purpose of this study was twofold: 1) to identify which inflammatory markers are associated with bone mass in children with overweight/obesity and, 2) to examine whether this association varies according to muscular fitness levels in this population.

Material and methods

Participants and study design

The present cross-sectional study was developed within the ActiveBrains project framework (ClinicalTrial.gov ID: NCT02295072). A detailed description of the study

design, purpose, methodology and inclusion/exclusion criteria has been published elsewhere (187). The ActiveBrains project measured 110 prepubertal children with overweight/obesity from Granada (Southern Spain). Participants were recruited from the Paediatric Unit of the University Hospitals San Cecilio and Virgen de las Nieves. The study protocol was approved by the Review Committee for Research Involving Human Subjects at the University of Granada (Reference: 848, February 2014) and informed consent was obtained from parents.

In this report, a total of 55 children (10.2±1.2 years, 38 boys) with complete data on inflammatory markers, body composition (i.e. bone, FM and LM), objectively measured muscular fitness, and sexual maturation assessment were included (see flowchart in **Figure 1**).

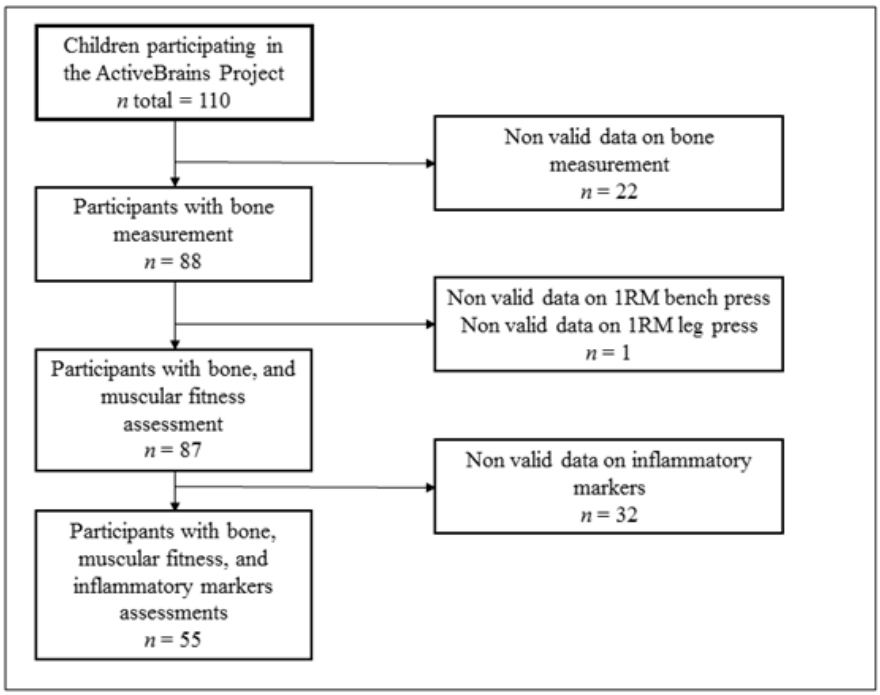

Figure 1. Flowchart of study participants. *DXA*, dual-energy x-ray absorptiometry; *1RM*, one repetition maximum

Anthropometry and sexual maturation

Body mass (kg) was measured with an electronic scale (SECA 861, Hamburg, Germany). Height (cm) and sitting height were measured with a precision stadiometer (SECA 225, Hamburg, Germany). BMI was calculated as: body mass (kg)/height (m^2) and the participants were classified into BMI categories according to the World Obesity Federation criteria (188).

Somatic maturity offset was assessed as years from peak height velocity (PHV) from age, height and sitting height using validated algorithms for children (189).

Inflammatory markers

Venous blood samples were obtained between 08:00 a.m. and 09:00 a.m. by venipuncture after an overnight fast (at least 12 hours) in all participants. Blood samples in tubes containing EDTA were spun immediately at 1000g for 10 min. Plasma was isolated and stored at -80°C until analyses. Three key cytokines analyzed in plasma were included in this study: interleukin-6 (IL-6, pg/mL), interleukin-1β (IL-1β, pg/mL), and tumour necrosis factor-α (TNF-α, pg/mL). IL-6, IL-1β and TNF-α were quantified by multiple analyte profiling technology (MILLIPLEX® MAP Human High Sensitivity T Cell Magnetic Bead Panel, EMD Millipore Corporation, Missouri, USA) with a kit plex (HCYIL6-MAG Anti-Human IL-6 Beads set, HCYIL1B-MAG Anti-Human IL-1β Bead, and HCYTNFA-MAG Anti-Human TNF-α Beads set), using one 96-Well plate with sealers (Cat. HSTCMAG-28SK). The intra- and inter-assay precision coefficients of variation for IL-6 were 5% and 20%, respectively, and sensitivity was 0.11 pg/mL. For both, IL-1β and TNF-α the intra- and inter-assay precision coefficients of variation were 5% and 15%, respectively, with a sensitivity of 0.14 pg/mL for IL-1β, and of 0.16

pg/mL for TNF-α. CRP (mg/L) was determined by turbidimetry (AU2700 Olympus Analyzer; Olympus UK Ltd, Watford, UK) with a sensitivity of 0.007 mg/L and inter-assay coefficients of variation 1.9%.

Two growth factors were analyzed by multiple analyte profiling technology (MILLIPLEX® MAP Human Angiogenesis/Growth Factor Magnetic Bead Panel 1, EMD Millipore Corporation, Missouri, USA) with a kit plex (HVEGF-MAG Anti-Human VEGF-A Bead, and HAGEGF-MAG Anti-Human EGF Bead), using one 96-Well plate with sealers (Cat. HAGP1MAG-12K). The intra- and inter-assay precision coefficients of variation for vascular endothelial growth factor A (VEGF, pg/mL) were 3.5% and 10%, respectively, and sensitivity was 8.1 pg/mL. For EGF (pg/mL), the intra- and inter-assay precision coefficients of variation were 3.2% and 6.8%, respectively, with a sensitivity of 1.0 pg/mL.

Body composition

Children were scanned with dual-energy X-ray Absorptiometry (DXA) using the Hologic Discovery Wi (Hologic Series Discovery QDR, Bedford, MA, USA). The DXA equipment was calibrated at the start of each testing day by using a lumbar spine phantom as recommended by the manufacturer. All DXA scans and analyses were performed using the APEX software (version 4.0.2) and were completed following the same protocol by the same researcher. The positioning of the participants and the analyses of the results were undertaken following recommendations from the International Society of Clinical Densitometry (190). The total body scan was used to obtain FM, LM, and BMC at the total body less head (TBLH) and at the LS.

Objectively measured muscular fitness

Muscular fitness was evaluated in laboratory conditions. We determined each participant's 1 repetition maximum (1RM) when the child was able to lift throughout the full range of motion in bench press and leg press tests (191). Participants received familiarization sessions before the testing session in order to ensure an adequate technique (i.e. controlled movements and proper breathing). Before attempting 1RM, participants performed 6 repetitions with a light load and 3 repetitions with a heavier load (50–90% estimated 1RM). Then, a series of single repetitions with increasing loads (0.5–2.3 kg for bench press and 10–20 kg for leg press) were performed. The 1RM was determined when participants fell short of the full range of motion on at least two non-consecutive attempts. A resting time of 3–5 minutes between attempts was allowed. Rate of perceived exertion at each attempt was obtained using the children's OMNI-Resistance Exercise scale (192). Moreover, during all testing procedures researchers obtained more information from the participants by asking questions such as: "How do you feel?", "Is the load light, medium or heavy?" and "Could you lift more?" to aid in the progression of the 1RM trials.

A muscular fitness score was computed by combining the standardized values of 1RM bench press and 1RM leg press tests. Each of these variables was standardized as follows: standardized value=(value−mean)/SD. The muscular fitness z-score was calculated as the mean of the 2 standardized scores (1RM bench press and 1RM leg press).

Statistical analyses

Data were analyzed using SPSS IBM statistics (version 20 for Windows, Chicago, IL) and the normal distribution of the raw variables was confirmed using visual check of histograms, Q-Q and box plots. Statistical significance was defined as $P<0.05$. Interaction analyses were performed between sex and inflammatory markers on the outcomes. No significant interactions were found ($P>0.05$), so analyses were carried out for boys and girls together.

Descriptive characteristics of participants are presented as mean ± standard deviation (SD). Stepwise hierarchical regression analyses were carried out to identify the inflammatory markers that best predicted bone mass. Sex, years from PHV and TBLH LM were considered for entry into step 1 of the model, and subsequent addition of inflammatory markers in step 2 was conducted to determine the contribution to the bone mass variables following step 1 adjustments. These covariates were selected because of their known association with bone mass (176). The standardized regression coefficients (β) are reported, and the squared semi-partial correlation coefficients (sr^2) were used to determine the contribution of each predictor in the overall variance of the model after removing shared contributions with other predictors. Collinearity was checked for the variables using the variance inflation factor and tolerance levels.

Finally, multiple linear regression analysis with interaction effect was used to test the role of muscular fitness in the association between inflammatory markers (those that were significant in the stepwise regression models) and bone mass. The interaction effects of muscular fitness in the association between inflammatory markers and bone mass were further examined (in those with $P<0.20$), stratifying by

high/low (above/below sex-, age-, and study-specific median) levels of muscular fitness.

Results

Descriptive characteristics are presented in **Table 1** (mean ± SD). The mean age of the participants was 10±1.2 years and they were 2.3±1.0 years below PHV, overweight and obesity was evident in 30.9% and 69.1% of them, respectively.

Table 1. Descriptive characteristics of the study sample

	All (*n* = 55)	Boys (*n* = 38)	Girls (*n* = 17)
Age (years)	10.2 ± 1.2	10.3 ± 1.2	9.9 ± 1.2
Height (cm)	144.5 ± 8.8	144.9 ± 7.4	143.5 ± 11.5
Body mass (kg)	56.2 ± 11.3	56.9 ± 10.4	54.4 ± 13.3
BMI ($kg \cdot m^{-2}$)	26.7 ± 3.7	26.9 ± 3.7	26.0 ± 3.6
Overweight (%)	30.9	28.9	35.3
Obesity (%)	69.1	71.1	64.7
Years from PHV (years)	-2.3 ± 1.0	-2.6 ± 0.8	-1.6 ± 1.2
Inflammatory markers			
IL-1β ($pg \cdot mL^{-1}$) [a]	1.7 ± 0.9	1.6 ± 0.9	1.9 ± 0.7
IL-6 ($pg \cdot mL^{-1}$) [a]	1.8 ± 1.3	1.8 ± 1.4	1.8 ± 1.0
TNF-α ($pg \cdot mL^{-1}$) [a]	4.1 ± 1.6	4.1 ± 1.7	3.9 ± 1.3
EGF ($pg \cdot mL^{-1}$) [a]	9.3 ± 20.8	7.9 ± 18.0	12.3 ± 26.5
VEGF ($pg \cdot mL^{-1}$) [a]	55.1 ± 51.1	49.0 ± 41.7	68.5 ± 67.1
CRP ($mg \cdot L^{-1}$) [a]	3.3 ± 3.1	3.6 ± 3.5	2.4 ± 1.6
Body composition			
TBLH BMC (g) [a]	988.42 ± 197.87	998.50 ± 186.18	965.88 ± 226.29
LS BMC (g) [a]	25.01 ± 6.18	24.03 ± 5.75	27.22 ± 6.71
TBLH LM (kg) [a]	27.3 ± 5.2	27.7 ± 4.5	26.6 ± 6.5
TBLH FM (kg) [a]	22.4 ± 6.4	22.8 ± 6.3	21.6 ± 6.9
Objective muscular fitness			
1RM bench press (kg)	22.1 ± 4.4	22.8 ± 4.7	20.4 ± 3.2
1RM leg press (kg)	138.2 ± 26.4	139.1 ± 26.6	136.1 ± 26.4
Muscular fitness z-score [†]	0.0 ± 1.0	0.1 ± 1.1	-0.3 ± 0.8

Data are presented as mean ± standard deviation.

[a] Values were Blom-transformed before analysis, but non-transformed values are presented

[†] Z-score mean computed from 1RM bench press (kg) and 1RM leg press (kg) tests

BMI body mass index; *PHV* peak height velocity; *IL* interleukin; *TNF-α* tumour necrosis factor alpha; *EGF* epidermal growth factor; *VEGF* vascular endothelial growth factor A; *CRP* c-reactive protein; *TBLH* total body less head; *LS* lumbar spine; *BMC* bone mineral content; *LM* lean mass; *FM* fat mass; *1RM* one maximum repetition

Table 2 shows the stepwise multiple regression analyses for identifying the inflammatory markers that explained the variance in the outcome variables in children with overweight/obesity. The probability of F-to-remove ≥ 0.1 was established in order to identify these markers. For TBLH BMC, 88% of the variance was explained by TBLH LM, years from PHV, IL-6, sex, and VEGF (sr^2 = 0.009 – 0.135), whilst 66% of the variance in LS BMC was explained by TNF-α, years from PHV, TBLH LM, IL-1β, and sex (sr^2 = 0.001 – 0.096).

The role of muscular fitness z-score in the association of inflammatory markers (those previously included in the stepwise method) and bone mass is shown in **Table 3**. After adjusting for sex, years from PHV, and TBLH LM, the interaction effect of muscular fitness showed a positive trend in the association of VEGF with TBLH BMC (P = 0.122) and TNF-α with LS BMC (P = 0.057). No evidence of interaction with muscular fitness was found in the remaining associations of IL-6 with TBLH BMC (P = 0.857) and IL-1β with LS BMC (P = 0.309).

Table 2. Stepwise hierarchical regression models to identify the inflammatory markers that best predict bone mass in
(*n* = 55)

Outcome	Predictors	*β STD*	sr^2	*P*	Outcome	Predictors
TBLH BMC	Sex	-0.140	0.009	**0.038**	LS BMC	Sex
R^2 *adj* = 0.88	Years from PHV	0.376	0.039	**<0.001**	R^2 *adj* = 0.66	Years from PHV
	TBLH LM	0.637	0.135	**<0.001**		TBLH LM
	IL-6	-0.136	0.019	**0.006**		TNF-α
	VEGF	-0.099	0.009	**0.040**		IL-1β

IL-1β, IL-6, TNF-α, EGF, VEGF and CRP were introduced in the step 2, but only those that were included by the stepw
indicates $P < 0.050$
β STD is the estimated standardized regression coefficient of the focal fitness test
sr^2 = Semi-partial correlation coefficients reflecting inflammatory explanatory value after accounting for the other va
PHV peak height velocity; *IL* interleukin; *TNF-α* tumour necrosis factor alpha; *EGF* epidermal growth factor; *VEGF* vas
CRP C-reactive protein; *TBLH* total body less head; *LS* lumbar spine; *BMC* bone mineral content; *LM* lean mass

Table 3. Multiple linear regression analyses with interaction effect for testing the role of muscular fitness in the asso
markers and bone mass variables in children with overweight/obesity (n = 55)

Outcome	Predictors	*β STD*	*P-value*	Outcome	Predictors
TBLH BMC	Sex	-0.135	0.057	LS BMC	Sex
R^2 *adj* = 0.87	Years from PHV	0.385	**<0.001**	R^2 *adj* = 0.55	Years from PHV
	TBLH LM	0.581	**<0.001**		TBLH LM
	VEGF	-0.073	0.179		IL-1β
	MF z-score†	0.072	0.259		MF z-score†
	VEGF x MF	0.081	0.122 ǂ		IL-1β x MF
TBLH BMC	Sex	-0.141	**0.049**	LS BMC	Sex
R^2 *adj* = 0.88	Years from PHV	0.382	**<0.001**	R^2 *adj* = 0.64	Years from PHV
	TBLH LM	0.580	**<0.001**		TBLH LM
	IL-6	-0.126	**0.017**		TNF-α
	MF z-score†	0.098	0.093		MF z-score†
	IL-6 x MF	0.009	0.857		TNF-α x MF

Boldface indicates $P < 0.050$
ǂ P interaction < 0.20
β STD is the estimated standardized regression coefficient of the focal fitness test
† Z-score mean computed from 1RM bench press (kg) and 1RM leg press (kg) tests
PHV peak height velocity; *TBLH* total body less head; *LS* lumbar spine; *BMC* bone mineral content; *LM* lean mass; *MF* m
endothelial growth factor A; *IL* interleukin; *TNF-α* tumour necrosis factor alpha

Figure 2 shows the standardized β regression slopes of VEGF with TBLH BMC and TNF-α with LS BMC, according to muscular fitness levels. Stratified analyses by muscular fitness levels (below/above median) showed a significant inverse association between VEGF and TBLH BMC in the low muscular fitness group (**Figure 2A**, β = -0.152, P = 0.032), whilst no evidence of association was found in the high muscular fitness group (**Figure 2A**, β = -0.045, P = 0.598). Likewise, an inverse association between TNF-α and LS BMC was found in the low muscular fitness group (**Figure 2B**, β = -0.491, P < 0.001), although this association was non-significant in the high muscular fitness group (**Figure 2B**, β = -0.060, P = 0.666).

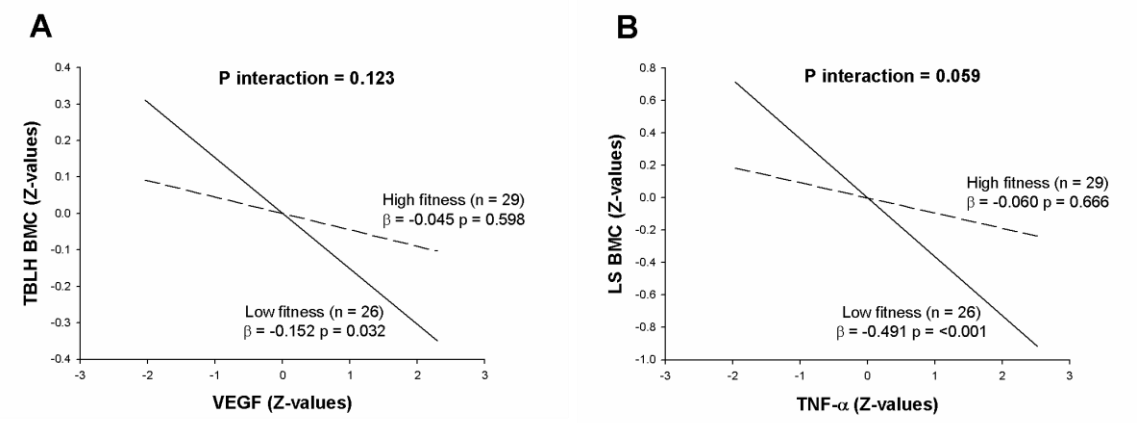

Figure 2. Graphical representation of the standardized regression slopes between VEGF and TBLH BMC by levels of muscular fitness (A); and between TNF-α and LS BMC by levels of muscular fitness (B). High/low fitness groups were defined as being above/below the age, sex and study-specific median values for average muscular fitness z-score†. The regression models were adjusted for sex, years from PHV, and TBLH LM. The standardized coefficients are interpreted as the number of SDs that the outcome changes as a result of 1-SD change in the predictor.
† Z-score mean computed from 1RM bench press (kg) and 1RM leg press (kg) tests
PHV peak height velocity; *VEGF* vascular endothelial growth factor A; *TNF-α* tumour necrosis factor alpha; *TBLH* total body less head; *BMC* bone mineral content; *LM* lean mass

Discussion

In the present study we showed IL-6 and VEGF to be associated with TBLH BMC and TNF-α and IL-1β with LS BMC in children with overweight/obesity. In addition, our results suggested that higher levels of muscular fitness may attenuate the adverse effects of VEGF and TNF-α on TBLH BMC and LS BMC, respectively. To the best of our knowledge, this is one of the few studies which thoroughly addresses the influence of inflammatory markers on bone mass, and the first study examining the role of muscular fitness in the relationship between inflammatory markers and bone mass.

Inflammatory markers and bone mass in overweight/obese children

In this study, an inverse association between VEGF and TBLH BMC was found after controlling for the effect of sex, years from PHV, and TBLH LM. Our results were comparable to a longitudinal study in which serum VEGF was inversely associated with BMC/height at the total body in boys with overweight whose BMI gain was higher during pubertal years (90). Elevated circulating levels of VEGF have been found in obese population as hypoxia-induced by adipose tissue expansion produce VEGF (193). However, VEGF functions on bone development depend both on autocrine and paracrine pathways. For instance, VEGF stimulates osteoblast differentiation and inhibits adipocytes differentiation via intracrine pathway, whereas osteoblast-derived VEGF leads osteoclast differentiation via paracrine pathway (194). In the light of these findings, we could speculate that despite VEGF concentrations are important for the adipose tissue vascularization in this population, highly-expressed VEGF as a consequence of the overweight/obese condition might have detrimental effects on

bone mass accumulation, possibly explained by the dysregulation of autocrine and paracrine mechanisms.

IL-6 was also inversely associated with TBLH BMC after controlling for the same set of cofounders. Our results are in accordance with Hanks et al. (89) who found a negative correlation between IL-6 and BMC in prepubertal girls. Similarly, Mengel et al. (90) found that the changes in serum IL-6 were negatively correlated with LS aBMD in boys with overweight and extensive BMI gain during the pubertal years. However, they did not find any association between IL-6 and LS aBMD after controlling for the effect of testosterone, body fat percentage, and BMI. Previous studies have reported that IL-6 directly promotes osteoclastogenesis by binding with receptors on pre-osteoclasts or indirectly alters bone remodelling by inducing JAK/STAT3 pathways through osteoblasts and secrete pro-osteoclasts mediators (i.e. receptor activator of nuclear factor kappa-B ligand [RANKL], and IL-1) (98). Overall, our findings agree with the idea that IL-6 have anti-osteogenic and pro-osteoclastic effects on bone and these effects might already be present in prepubertal children with overweight/obesity.

Like IL-6, *in vitro* studies have also reported the osteoclastogenic role of TNF-α (101). Otherwise, few studies have documented this role in humans. Zheng et al (95) reported that TNF-α produced by stimulated whole blood cells was inversely associated with LS aBMD in postmenopausal women, whereas Ding et al (102) found that the inverse association between serum TNF-α and LS aBMD in older men disappeared after controlling for IL-6. Our results indicate that plasma levels of TNF-α were inversely associated with LS BMC independently on sex, years from PHV, and TBLH LM. Besides, IL-6 did not come up as a predictor of LS BMC in the stepwise multiple linear regression.

IL-1β is a proinflammatory marker associated with osteoclastogenesis via induction of RANKL and inhibition of osteoprotegerin and reduces osteoblast recruitment *in vitro* (94). Unexpectedly our results suggest, for the first time, a positive association between IL-1β and LS BMC after controlling for potential cofounders in children with overweight/obesity. This finding agrees with the study of Pacifici et al. (195) in which an increased IL-1 production was associated with bone formation in adults independently on sex and menopausal status. This can be explained by the fact that short stimulation of mesenchymal stem cells with IL-1β leads to osteogenic differentiation through upregulation of genes in MG63-GFP osteoblasts (196). On the contrary, IL-1β has been negatively associated with LS aBMD in postmenopausal women (95) and Mengel et al. (90) did not find significant associations between IL-1β and aBMD in overweight children.

Muscular fitness, inflammatory markers and bone mass in overweight/obese children

Bone marrow is a complex environment, in which a variety of cell types (i.e. blood cells, osteoblasts, osteoclasts, and adipocytes) share a common space locally releasing cytokines and growth factors that could affect the cells in their proximity (177). Furthermore, bone development is regulated by modelling and remodelling processes that depend on the mechanical forces applied by the muscles to the skeleton (197). Our study suggests that higher levels of muscular fitness might attenuate the detrimental effects of VEGF and TNF-α on TBLH BMC and LS BMC, respectively (Figure 2). In this regard, partial correlations controlling for sex and years from PHV showed that VEGF was negatively correlated with muscular fitness (data not shown, r = -0.36, P = 0.008), although no evidence of correlation was found between TNF-α and muscular fitness

(data not shown, r = -0.01, P = 0.923). The latter result contrasts with Steene-Johannessen et al. (185) who found a negative correlation between TNF-α and muscular fitness in prepubertal children after controlling for pubertal stage. Nevertheless, the beneficial effect of muscular fitness on bone development is well documented in growing children (198). Moreover, the association between muscular fitness and bone mass is mediated by LM in prepubertal children (144). Likewise, an inhibitory effect of LM on obesity-related inflammation has been suggested in middle-aged adults (199). Thereby, our findings agree with literature and support the fact that there is a crosstalk between adipocytes and myocytes interacting with obesity and its related disorders even in children with overweight/obesity. We speculate that the detrimental consequences of excessive FM (i.e., inflammation) in children with overweight/obesity could be counteracted, to some extent, by maintaining optimal levels of muscular fitness.

Strengths and limitations

Some limitations need to be considered. At first, our cross-sectional design rules out the possibility of identifying cause-effect relationships. Secondly, the number of participants with complete data in all studied variables is relatively small, but similar to previous studies (90,91). Thirdly, our study has used plasma samples to measure inflammatory markers. Previous studies have used plasma or serum samples and therefore, comparisons may be affected. However, as shown in a recent study the correlations between plasma and serum measurements suggest that the differences in metabolite concentrations does not necessarily introduce a bias in cross-sectional studies (200). Notwithstanding, the use of DXA and the accuracy of the objective

methodology used for muscular fitness and blood measurements are strengths of this study.

Conclusion

In summary, our findings suggest that the link between obesity and bone health may be at least explained by inflammatory mechanisms in children with overweight/obesity. Specifically, IL-6 and VEGF were negatively associated with TBLH BMC, whereas TNF-α (negatively) and IL-1β (positively) were associated with LS BMC. Furthermore, our data suggest that high levels of objectively measured muscular fitness may attenuate the adverse effects of VEGF and TNF-α on TBLH BMC and LS BMC, respectively. In the light of these findings, appropriate levels of muscular fitness may preserve normal bone accretion in this population. Future longitudinal and intervention studies in this population are needed to confirm these findings.

Study 2

Adipokines and bone outcomes in children with overweight/obesity: The moderator role of muscular fitness.

Introduction

Obesity and osteoporosis are global epidemics that warrant increased attention by paediatricians and other health care professionals. Childhood obesity is not a direct cause of osteoporosis, but massive fat accumulation and its related consequences (i.e., low-grade chronic inflammation) could impair the bone acquisition during the growing years (22), with a higher risk for fractures later in life (201).

A growing body of evidence suggests that children with overweight/obesity tend to have higher bone mineral content (BMC) than their normal-weight peers, indicating that the body mass exerts a positive effect on bone structure (20). Notwithstanding, the increased rate of fractures in this population suggests a poorer bone quality (23). Bone structure and quality depend, among other factors, on the effects of circulating inflammatory markers (i.e., cytokines and growth factors), immunomodulators (i.e., adipokines) and mechanical stimulation (i.e., overload and physical activity) (87). However, to the best of the authors´ knowledge, only one study so far has included adipokines (e.g., leptin and adiponectin) as predictors of bone outcomes in children with overweight/obesity (92).

Leptin seems to play various roles in bone metabolism through two pathways. In the central pathway, leptin leads to bone loss by either suppressing osteoblast proliferation or increasing the expression of the receptor of NF-κB ligand (RANKL) which promotes the resorption of the osteoclasts (114). In the peripheral pathway, leptin increases bone mass by enhancing the proliferation and differentiation of bone marrow mesenchymal stem cells into osteoblastic lineage (115). By contrast, the role of the adiponectin on bone metabolism requires further investigation. Recent data indicate that adiponectin acts to promote osteoblastogenesis within the bone marrow

niche and simultaneously inhibiting osteoclastogenesis, although these mechanisms might be dysregulated in obese individuals (120).

Optimal muscular fitness levels during childhood and adolescence are paramount for future bone health (145). In this sense, previous cross-sectional studies reported that the association between muscular fitness and bone outcomes was explained by lean mass (LM) in different growth stages (181,182). Following the mechanostat theory, larger muscles exert greater forces on the bones, which will adapt and therefore improve their strength (202). Furthermore, recent evidence highlights that both relative handgrip and standing long jump are negatively associated with leptin in children, whereas no association was found between adiponectin and any of the muscular fitness components after controlling for sex, maturity and body mass index (BMI) (203). Similarly, Steene-Johannessen et al. (185) reported that higher muscular fitness (i.e., standardized score of handgrip, standing long jump, sit-up test and Biering–Sørensen test) was associated with lower levels of leptin in children, whereas no association was found with adiponectin after controlling for adiposity and cardiorespiratory fitness.

Recently, we provided further insight in the relationship between inflammatory markers and bone outcomes in children with overweight/obesity with different levels of muscular fitness (204). Given the low-grade systemic inflammation comprises many overlapping events, we investigated adipokines and inflammatory markers as consecutive events to facilitate understanding. Therefore, the aim of the present study was twofold: 1) to examine the association between adipokines (i.e., leptin and adiponectin) and bone outcomes in children with overweight/obesity and 2) to check whether muscular fitness (i.e., upper body and lower body) moderate these associations.

Material and methods

Study design and participants

This cross-sectional study used baseline data from the ActiveBrains project (http://profith.ugr.es/activebrains). The trial protocol has been described elsewhere (187). A total of 110 children with overweight/obesity aged 8 to 11 years were recruited from the Paediatric Unit of the "San Cecilio" and "Virgen de las Nieves" University Hospitals (Granada, Spain) and through advertisements in local media and school contacts. The inclusion criteria were as follows: (1) to be children with overweight or obesity based on the World Obesity Federation cut-off points, (2) to be 8 to 11 years old, (3) not to have any physical disabilities or neurological disorder that affects their physical performance, and (4) in the case of girls, not to have started the menstruation at the moment of the assessments. In this research, we included 85 children (9.9 ± 1.2 years old; 38% girls) with complete data on adipokines (i.e., circulating leptin and adiponectin), muscular fitness (i.e., upper body and lower body), body composition (i.e., bone, fat and lean mass). The baseline data collection was divided in three waves and took part from November 2014 to February 2016.

A participant information sheet was given to the parents or legal guardians and a written informed consent was obtained from both the guardian. The ActiveBrains project was approved by the Ethics Committee on Human Research of the University of Granada and was registered in ClinicalTrials.gov (identifier: NCT02295072).

Anthropometry and somatic maturity

The body mass was measured to the nearest 0.1 kg using an electronic scale (SECA 861, Hamburg, Germany). The height was measured to the nearest 0.1 cm using a

precision stadiometer (SECA 225, Hamburg, Germany). The body mass index (BMI) was calculated as body mass/height (kg/m^2) and the participants were classified as children with overweight or obesity according to sex- and age- specific cut-off points defined by Cole et al. (188).

The years from peak height velocity (PHV) was used as maturational landmark and was predicted through age and anthropometric measures (height in girls, and sitting height in boys) using validated algorithms for boys and girls (189).

Adipokines

Blood samples were obtained between 8:00 am and 10:00 am by venipuncture after an overnight fast. The blood samples were drawn into tubes with EDTA and was spun immediately (1000 x g for 10 min at 4°C). Plasma was isolated, aliquoted, and stored at -80°C in the Centre of Biomedical Research (Granada, Spain) until analysis.

Plasma leptin concentrations were quantitatively determined in duplicates by the Luminex IS 100/200 system (Luminex Corporation, Austin, TX), with the xMAP technology (MILLIPLEX® MAP, Human Angiogenesis/Growth Factor Magnetic Bead Panel 1, EMD Millipore Corporation, Missouri, USA) with a kit plex (HCCLPTN-MAG Anti-Human Leptin Bead). The intra- and inter-assay precision coefficients of variation for leptin were 2.8% and 6.0%, and sensitivity was 42.8 pg/mL.

Plasma adiponectin concentrations were quantitatively determined in duplicates by the Luminex IS 100/200 system (Luminex Corporation, Austin, TX), with the FLEXMAP 3D technology (MILLIPLEX® MAP, Human Adipokine Magnetic Bead Panel 1, EMD Millipore Corporation, Missouri, USA) with a kit plex (HADK1MAG-61K Human Adipokine

Magnetic Bead). For adiponectin, the intra- and inter-assay precision coefficients of variation were <10% and <15%, and sensitivity was 11 pg/mL.

Body composition and bone outcomes

Bone mass, fat mass and lean mass [body mass - (fat mass + bone mass)] were measured by dual-energy x-ray absorptiometry (DXA) using the Hologic Discovery Wi (Hologic Series Discovery QDR, Bedford, MA, USA). DXA equipment was calibrated at the start of each testing day by using a lumbar spine phantom as recommended by the manufacturer. The APEX software (version 4.0.2) was used to analyse the scans following the recommendations for children and adolescents (190). The total body scan was used to obtain fat mass (FM, kg), lean mass (LM, kg), bone mass content (BMC, kg) and areal bone mineral density (aBMD) at the total body less head (TBLH) and lumbar spine (LS).

Objectively measured muscular fitness

Upper-body and lower-body muscular fitness were assessed in laboratory conditions. We determined each participant's 1 repetition maximum (RM) when the child was able to lift throughout the full range of motion in bench press and leg press tests (191). Participants received familiarization sessions before the testing session in order to ensure an adequate technique (i.e., controlled movements and proper breathing). Before attempting RM, participants performed six repetitions with a light load and three repetitions with a heavier load (i.e., 50–90% estimated RM). Then, a series of single repetitions with increasing loads (i.e., 0.5–2.3 kg for bench press and 10–20 kg for leg press) were performed. The RM was determined when participants fell short of

the full range of motion on at least two non-consecutive attempts. A resting time of 3–5min between attempts was allowed. Rate of perceived exertion at each attempt was obtained using the children's OMNI-Resistance Exercise scale (192). Moreover, during all testing procedures researchers obtained more information from the participants by asking questions such as: "How do you feel?", "Is the load light, medium or heavy?" and "Could you lift more?" to aid in the progression of the RM trials.

Statistical analysis

Data were analysed using SPSS IBM statistics (version 20 for Windows, Chicago, IL) and statistical significance was defined as $P<0.05$. Descriptive characteristics of participants are presented as mean ± standard deviation (SD) for continuous variables and percentages for categorical variables. All variables were checked for normality using visual check of histograms, Q-Q and box plots. Skewed data were log-transformed for analytical purposes. Sex interaction was checked in the association of adipokines (i.e., leptin and adiponectin) with bone outcomes (i.e., TBLH BMC and LS BMC). No significant interactions were found (all $P>0.2$), so analyses were performed for boys and girls together.

Multiple regression analyses were carried out to examine the association between adipokines and bone outcomes. Sex, years from PHV and TBLH LM were introduced as covariates based on their known association with bone outcomes (205). The standardized regression coefficients (β) are reported, and the squared semi-partial correlation coefficients (sr^2) were used to determine the contribution of each predictor in the overall variance of the model after removing shared contributions with other

predictors. Collinearity was checked for the variables using the variance inflation factor and tolerance levels.

Multiple linear regression analysis with interaction effect was used to test the role of muscular fitness in the association between adipokines (those that were borderline in the multiple regression models) and bone outcomes. The interaction effects of muscular fitness in the association between adipokines and bone outcomes were further examined (in those with $P < 0.20$), stratifying by high/low (above/below study-specific median) levels of muscular fitness. Finally, moderation analyses were conducted using the PROCESS macro 3.1 (147) in order to provide greater resolution for clarifying interactions. PROCESS uses ordinary least squares regression analysis when predicting continuous variables (bone outcomes in the current study) and a bias-corrected bootstrap method (with 5000 bootstrapped samples) to estimate the conditional (moderated) effects. In this regard, the Johnson-Neyman technique was used to test for significance along a continuous moderator variable and delineates the slope of the relationship at each value. In the context of the current study, the technique seeks for specific muscular fitness cut points in which the significant relationship between adipokines and bone outcomes disappears. The moderation analyses were adjusted for sex, years from PHV and TBLH LM.

Results

The descriptive characteristics of the children participating in the study are shown in **Table 1**. The mean age of the participants was 10.0±1.2 years and they were -2.3 ± 1.0 years from PHV. Overweight and obesity was evident in 26.2% and 73.8% of them, respectively.

Table 1. Descriptive characteristics of the sample (mean ± standard deviation)

	All (*n* = 84)	Boys (*n* = 53)	Girls (*n* = 31)
Age (years)	10.0 ± 1.2	10.2 ± 1.2	9.7 ± 1.1
Years from PHV (years)	-2.3 ± 1.0	-2.7 ± 0.9	-1.8 ± 1.0
Height (cm)	143.8 ± 8.6	144.2 ± 7.9	142.9 ± 9.8
Body mass (kg)	55.3 ± 10.9	55.9 ± 10.5	54.2 ± 11.6
BMI (kg/m^2)	26.5 ± 3.5	26.7 ± 3.4	26.3 ± 3.6
Overweight (%)	26.2	24.5	29.0
Obesity (%)	73.8	75.5	71.0
Body composition			
TBLH LM (kg) [a]	26.6 ± 5.0	27.2 ± 4.9	25.7 ± 5.2
TBLH FM (kg)	22.3 ± 6.2	22.2 ± 5.9	22.4 ± 6.8
Adipokines			
Leptin (ng/mL) [a]	11.1 ± 5.8	11.4 ± 5.8	10.6 ± 5.8
Adiponectin (µg/mL) [a]	7.1 ± 5.3	7.8 ± 5.9	6.0 ± 3.9
Bone outcomes			
TBLH BMC (g) [a]	961.11 ± 200.64	976.61 ± 206.05	934.60 ± 191.41
TBLH aBMD (g/cm^2) [a]	0.769 ± 0.058	0.774 ± 0.059	0.760 ± 0.056
LS BMC (g) [a]	24.86 ± 6.16	24.22 ± 6.17	25.94 ± 6.08
LS aBMD (g/cm^2) [a]	0.756 ± 0.082	0.737 ± 0.069	0.787 ± 0.093
Muscular fitness			
RM bench press (kg)	21.4 ± 4.4	22.4 ± 4.5	19.7 ± 3.8
RM leg press (kg)	134.9 ± 26.2	136.7 ± 28.2	132.0 ± 22.5

[a] Variables were log transformed for analytical purposes, but non-transformed variables are presented
BMI body mass index; *PHV* peak height velocity; *TBLH* total body less head; *LS* lumbar spine; *BMC* bone mineral content; *aBMD* bone mineral density; *LM* lean mass; *FM* fat mass; *RM* repetition maximum

The association between adipokines and bone outcomes is shown in **Table 2**. No significant association was found (all p ≥ 0.05), although the association between leptin and LS BMC was borderline after adjusting for sex, years from PHV and TBLH LM (β=-0.162, sr^2=0.022, P=0.053). The interaction effects of muscular fitness in the association of leptin and LS BMC are shown in **Table 3**. After adjusting for the same set of covariates, the interaction effect of RM leg press was borderline (p=0.074), whereas non-significant interaction was found with RM bench press (P=0.543).

STUDIES' METHODS, RESULTS AND DISCUSSION

Table 2. Multiple regression models to examine the association between adipokines and bone outcomes in children v

Outcome	Predictors	*β*	sr^2	*P-value*	Outcome	Predictors
TBLH BMC	Sex	-0.157	0.012	**0.009**	LS BMC	Sex
R^2 *adj* = 0.86	Years from PHV	0.390	0.041	**<0.001**	R^2 *adj* = 0.52	Years from PHV
	TBLH LM	0.633	0.114	**<0.001**		TBLH LM
	Leptin	-0.033	0.001	0.455		Leptin
TBLH BMC	Sex	-0.137	0.001	**0.029**	LS BMC	Sex
R^2 *adj* = 0.85	Years from PHV	0.387	0.041	**<0.001**	R^2 *adj* = 0.49	Years from PHV
	TBLH LM	0.622	0.125	**<0.001**		TBLH LM
	Adiponectin	0.023	0.001	0.599		Adiponectin
TBLH aBMD	Sex	-0.107	0.005	0.279	LS aBMD	Sex
R^2 *adj* = 0.61	Years from PHV	0.271	0.019	**0.042**	R^2 *adj* = 0.32	Years from PHV
	TBLH LM	0.597	0.102	**<0.001**		TBLH LM
	Leptin	-0.059	0.003	0.433		Leptin
TBLH aBMD	Sex	-0.118	0.007	0.234	LS aBMD	Sex
R^2 *adj* = 0.61	Years from PHV	0.286	0.023	**0.031**	R^2 *adj* = 0.32	Years from PHV
	TBLH LM	0.561	0.102	**<0.001**		TBLH LM
	Adiponectin	-0.016	0.000	0.825		Adiponectin

Boldface indicates $P < 0.050$; † Borderline *p-values* $0.05 < P < 0.10$

β is the estimated standardized regression coefficient; sr^2 = Semi-partial correlation coefficients reflecting adipok accounting for the other variables included in the model

PHV peak height velocity; *TBLH* total body less head; *LS* lumbar spine; *LM* lean mass; *BMC* bone mineral content; *aBMD*

Table 3. Multiple linear regression analyses with interaction effect for testing the role of muscular fitness in the a
bone outcomes in children with overweight/obesity

Outcome	Predictors	*β*	*P-value*	Outcome	Predictors
LS BMC	Sex	0.068	0.537	LS BMC	Sex
R^2 *adj* = 0.53	Years from PHV	0.428	**0.004**	R^2 *adj* = 0.53	Years from PHV
	TBLH LM	0.368	**0.012**		TBLH LM
	Leptin	-0.349	0.338		Leptin
	RM bench press	-0.036	0.909		RM leg press
	Leptin x RM bench press	0.284	0.543		Leptin x RM leg p

Boldface indicates $P < 0.050$

† Borderline *p-values* $0.05 < P < 0.20$

β is the estimated standardized regression coefficient

PHV peak height velocity; *TBLH* total body less head; *LM* lean mass; *BMC* bone mineral content; *LS* lumbar spine; *RM*

Figure 1 depicts the regression slopes of leptin with LS BMC, as a function of RM leg press. The results revealed a significant inverse association between leptin and LS BMC in the low RM leg press group (**Fig 1.** β=-0.314, P=0.022), whereas no evidence of association was found in the high RM leg press group (**Fig 1.** β=-0.046, P=0.693). **Figure 2** shows the regression slope estimates and the 95% confidence intervals for the association between leptin and LS BMC as a function of RM leg press. The Johnson-Neyman technique revealed that the significant inverse association between leptin and LS BMC became non-significant when RM leg press was above 133.3 kg (51.2% of sample).

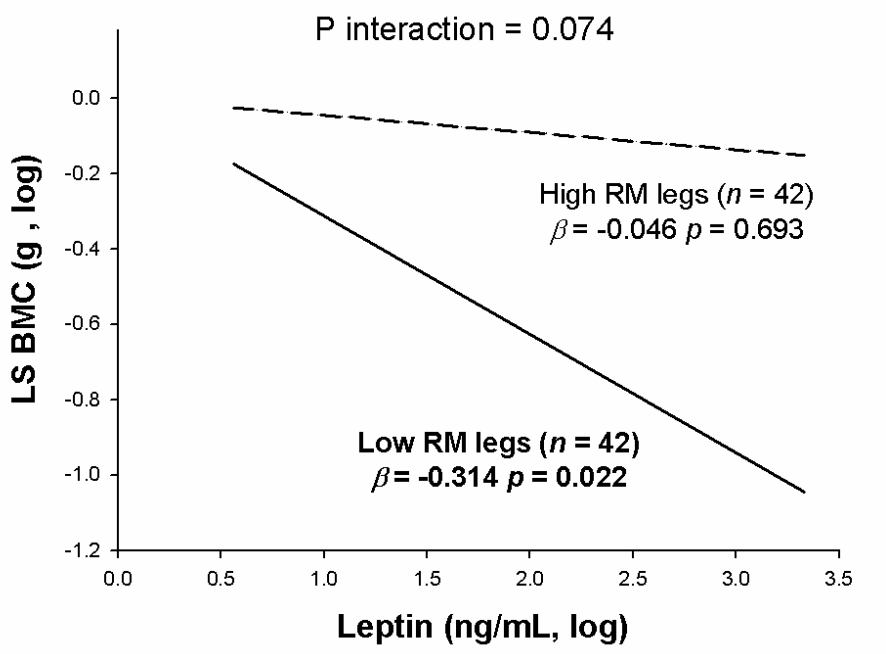

Figure 1. Graphical representation of the regression slopes between leptin and LS BMC by levels of RM leg press. Low and high levels were defined as being above/below the study-specific median value for RM leg press. The regression models were adjusted for sex, years from PHV and TBLH LM.
PHV peak height velocity; *TBLH* total body less head; *BMC* bone mineral content; *LS* lumbar spine; *LM* lean mass; *RM* repetition maximum

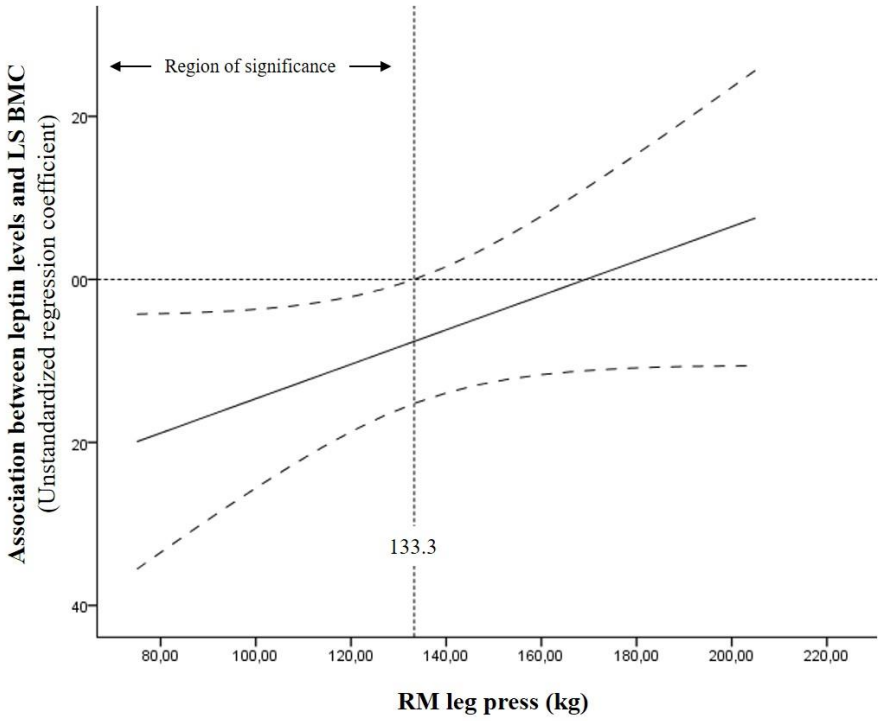

Figure 2. Regression slope estimate and 95% confidence interval for the association between leptin and LS BMC as a function of RM leg press, based on Johnson-Neyman results. The analyses were adjusted for sex, years from PHV and TBLH LM.
PHV peak height velocity; *TBLH* total body less head; *BMC* bone mineral content; *LS* lumbar spine; *LM* lean mass; *RM* repetition maximum

Discussion

The main findings of the present study are: 1) leptin and adiponectin levels were not associated with bone outcomes in children with overweight/obesity; 2) there was evidence for an interaction between leptin and RM leg press on LS BMC; 3) higher leptin levels were related to lower LS BMC in those children with overweight/obesity that had low absolute RM leg press. Likewise, those children whose RM leg press was above 133.3 kg could overcome the negative influence of leptin on LS BMC. To our knowledge, this is one of the few studies that thoroughly addresses the association between adipokines and bone outcomes in children with overweight/obesity, and the first study examining the role of muscular fitness in this relationship.

Our results showed a borderline negative association between leptin and LS BMC after adjusting for sex, years from PHV and TBLH LM. This finding agrees with those reported by Dimitri et al. (92) whom using peripheral quantitative computed tomography reported a negative correlation between leptin and various bone parameters (i.e., radial cortical porosity, radial cortical pore diameter, tibial trabecular thickness and tibial trabecular Von Mises stress) in children with obesity. Furthermore, evidence from adult population suggested that leptin is inversely associated with femoral bone outcomes in men and premenopausal women after controlling for age and BMI (206). Moreover, animal studies revealed that the effect of leptin on bone is dose-dependent (207), although this cause-effect relationship is not feasible to test in humans. In this sense, Meng et al. (112) recently implemented a two-sample mendelian randomization and found a negative association between leptin and LS bone outcomes. Given that leptin seems to have no effect on cortical bone and low circulating levels of leptin increases trabecular bone volume in mice (208), it is reasonable that leptin may affect the spine because of the greater trabecular bone proportion (209). With this in mind, our results reinforce the assumption that the negative effect of fat on bone is mediated by leptin levels even in the paediatric stage (210,211).

A few studies have examined the association between adiponectin and bone outcomes in children with overweight/obesity. The results of the present investigation showed no significant association between adiponectin and bone outcomes after adjusting for sex, years from PHV and TBLH LM. Corroborating with our findings, Dimitri et al. (92) found no significant association between adiponectin and bone outcomes in children with obesity, although they reported a negative association between adiponectin and Wnt-inhibitor Dikkopf-1 after skeletal maturation and sex adjustment

(212). Wnt-inhibitor Dikkopf-1 has been recently identified as a negative regulator of bone growth (213). Moreover, Campos et al. (121) found adiponectin to be negatively correlated with C-terminal telopeptide in adolescents with obesity, a marker released into the bloodstream during bone resorption. Overall, adiponectin might have an indirect effect of bone, although this effect may not be present in the paediatric stage yet. Further studies controlling for other relevant covariates (i.e., lean mass) will confirm it.

Importantly, we also took into account the interaction effect of muscular fitness (both absolute and relative) since the strain received by bone from muscular contraction and gravitational load is one of the most important factors of bone adaptation (128). In our study, high RM leg press eliminated the detrimental effect of leptin on LS BMC. Moreover, the role of RM leg press was evident from 133.3 kg. This result is in line with previous studies that found a negative association between muscular fitness and leptin levels in children (185,203,214). In this regard, heavier individuals have higher levels of RM leg press because they carry more body mass and therefore, higher muscular contractions are needed. According to our results, the minimum effective strain magnitude to enhance bone adaptation may be guaranteed when a high muscular fitness is acquired in children with overweight/obesity. In addition, we ran the analyses using field-based muscular fitness tests and we found similar results with the absolute handgrip strength and standing long jump (supplementary Table 1, Figure 1 and Figure 2), which reinforces the results of the present study.

Strengths and limitations

We acknowledge several limitations of our study. First, our findings are limited due to its cross-sectional design and causal direction cannot be inferred. Second, unconsidered confounding variables may also affect the findings based on observational data. Third, the number of participants with complete data in all studied variables is relatively small. Fourth, we used plasma samples to measure adipokines while previous studies have used plasma or serum samples and therefore, comparisons should be considered with caution. However, as shown in a recent study the correlations between plasma and serum measurements suggest that the differences in metabolite concentrations does not necessarily introduce a bias in cross-sectional studies (200). Fifth, the LS bone parameters were obtained from the whole-body scan and thus, caution should be used when comparing with LS bone parameters obtained from the regional scan. Nevertheless, children with overweight/obesity tend to have high levels of LS bone parameters and this fact reduces the difference between the whole-body and the regional scans (215). Sixth, peripheral quantitative computed tomography parameters were not assessed and therefore, we could not know if adipokines affects to the cortical or trabecular compartments. On the other hand, the use of DXA and the accuracy of the objective methodology used for muscular fitness and blood measurements are strengths of this study.

Conclusion

In summary, neither leptin nor adiponectin were associated with bone outcomes in children with overweight/obesity. Nevertheless, the interaction effect of RM leg press showed a trend in the association between leptin and LS BMC. Furthermore, our data

suggest that high levels of RM leg press may ameliorate (or even fully eliminate) the negative association observed between leptin and LS BMC in children with overweight/obesity. These results reinforce the importance of enhancing proper lower-body muscular fitness levels early in life. Therefore, school-based interventions aiming at improving muscular fitness (i.e., adequate frequency, volume and intensity according to pubertal stage) (216) are justified among children with overweight/obesity. Future longitudinal and intervention studies are needed to confirm these findings in this population.

Section 2

Mediator role of muscular fitness in the association between vitamin D and bone health.

Study 3

Muscular fitness mediates the association between 25-hydroxyvitamin D and areal bone mineral density in children with overweight/obesity

Introduction

The World Health Organization defines osteoporosis as a systemic skeletal disease characterized by low bone density and microarchitectural deterioration of bone tissue (217). Acquiring an optimal bone mineral accrual during childhood (i.e. late childhood and peripubertal years) is considered an important factor for reducing the risk of osteoporosis later in life (178). In general, children with overweight/obesity usually have greater areal bone mineral density (aBMD) than normal-weight children as they mature earlier, tend to be taller and have greater lean mass (178). Notwithstanding, Rokoff et al. (218) recently showed central adiposity to be inversely associated with aBMD Z-score at the total body less head (TBLH) in children with high levels of abdominal fat.

Childhood obesity is associated with a deficient 25(OH)D status in Spain (158). Vitamin D status is reflected by 25-hydroxyvitamin D [25(OH)D] levels and its concentration in children with obesity is influenced by vitamin D intake, season, ethnicity/race, decreased exposure to sunlight as a consequence of the sedentary lifestyle, or by 25(OH)D sequestration through adipose tissue (159). This prohormone is essential for bone development and remodelling processes, as well as for normal calcium and phosphorus homeostasis (149). Some studies evidenced that 25(OH)D-deficient children had lower aBMD Z-score at the lumbar spine (LS) and the total body, probably influenced by the consequent increase in parathormone levels (154,161).

Moderate-to-high muscular fitness at a young age is a powerful determinant of health (219). In this regard, Torres-Costoso et al. (144) found that children with good performance in handgrip and standing long jump had better and worse bone health, respectively. The latter associations were fully mediated by lean mass, whose function seems to be influenced by 25(OH)D levels (162). When calcitriol [$1,25(OH)_2D$, an active

metabolite of vitamin D] activates the nuclear vitamin D receptor (VDR), several slow pathways are activated leading to cytoskeletal protein synthesis important for muscle function (i.e. calmodulin, calbindin D-9K or insulin-like growth factor binding protein-3) (220–222). Moreover, the activation of the nuclear VDR also increases phosphate metabolism via increases in the uptake and accumulation of phosphate and ATP, resulting in positive effects on muscle contraction (223). In addition, the $1,25(OH)_2D$ activation of the membranous VDR stimulates rapid actions that affect Ca^{2+} handling and muscle cell proliferation and differentiation (224).

Although the relationship between 25(OH)D and muscular fitness has been described in youth, no study has jointly examined the association of these predictors with aBMD outcomes. Most published studies have been conducted using statistical multivariate procedures in order to control for potential confounders, but these statistical procedures are unable to distinguish between confounding and mediating variables. Mediation analysis allows us to clarify the process underlying the relationship between two variables and the extent to which this relationship can be modified or confounded by a third variable (225). Therefore, the aim of this study was to examine whether the relationship between 25(OH)D and aBMD outcomes is mediated by muscular fitness in children with overweight/obesity.

Material and methods

Design

A cross-sectional analysis was conducted of the baseline measurements of the ActiveBrains project (registered at Clinicaltrials.gov, number NCT02295072). A detailed description of the study has been published elsewhere (187). The ActiveBrains project

measured 110 children with overweight/obesity aged 8-11 years from Granada (south of Spain) according to the following inclusion criteria: 1) to be overweight or obese based on the World Obesity Federation (formerly named International Obesity Task Force) cut-off points 2) to be 8 to 11 years old, 3) not to have any physical disabilities or neurological disorder that affects their physical performance, and 4) in the case of girls, not to have started the menstruation at the moment of the assessments.

A total of 81 children with overweight/obesity (10.0±1.2 years old, 65% boys) with valid data on 25(OH)D, muscular fitness variables, body composition (i.e. bone, fat and lean mass) and sexual maturation were included in this report. Participants were recruited from the Paediatric Unit of the "San Cecilio" and "Virgen de las Nieves" University Hospitals in the province of Granada, Spain. Furthermore, we contacted with several schools of Granada and we advertised the study in the local media, inviting any child meeting the inclusion criteria. The study protocol was approved by the Ethics Committee on Human Research (CEIH) of the University of Granada (Reference: 848, February 2014). Written consent was obtained from parents for the participation of their children.

Anthropometrics and sexual maturation

Participants were weighted using an electronic scale (SECA 861, Hamburg, Germany) with an accuracy of 100 g. A precision stadiometer was used to assess height (cm) and sitting height (SECA 225, Hamburg, Germany) to the nearest 0.1 cm. BMI was calculated as: body mass (kg)/height (m^2) and the participants were classified as overweight or obese according to sex- and age-specific BMI cut-offs defined by Cole et al. (188).

Somatic maturity offset was assessed as years from peak height velocity (PHV) from age, height and sitting height using validated algorithms for children (189). In boys: -

8.128741 + (0.0070346 x (age x sitting height)), where R^2 = 0.906 and the standard error of the estimate = 0.514. In girls: -7.709133 + (0.0042232 × (age × height)), where R^2 = 0.898 and the standard error of the estimate = 0.528. PHV is the period of time of maximum growth in stature and therefore, years from PHV are considered in terms of time before and time after the PHV.

Vitamin D

Venous blood samples were obtained between 8:00 a.m. and 9:00 a.m. by venipuncture after an overnight fast (at least 12 hours) from September 2015 to February 2016 (Autumn and Winter). Blood samples in tubes containing EDTA were spun immediately at 30000g for 10 min. Plasma was isolated and stored at -80 °C until assayed. Plasma 25(OH)D was analysed by immunoturbidimetry (Alinity i 25-OH Vitamin D Reagent Kit ref. 08P4522, Abbot, Illinois, USA) with a sensitivity of 3.5 ng/mL and an intra-assay coefficient of variation of 2.5%.

Muscular fitness

Upper-body muscular fitness was assessed using the handgrip strength test through a dynamometer with adjustable grip (TKK 5101 Grip D, Takey, Tokyo Japan). Participants were instructed to squeeze continuously for ≥2 seconds with the elbow in full extension position. The test was repeated twice (right and left hands alternately). The best score of the 2 attempts for each hand was chosen and averaged (226). Finally, relative upper-body muscular fitness was expressed per kg of body mass (Handgrip strength [kg/kg]). Lower-body muscular fitness was assessed by the standing long jump test. Participants were instructed to push off vigorously and jump as far forward as possible, trying to land on

both feet. The distance reached was taken in centimetres from the take-off line and the heel of the nearest foot at landing. The longest attempt from 3 was recorded (cm). The scientific rationale for the selection of these tests, as well as their validity and reliability, has previously been demonstrated in children and adolescents (226).

A muscular fitness score (muscular fitness z-score) was computed by combining the standardized values of handgrip strength (kg/kg) and standing long jump (cm). Each of these variables was standardized as follows: z-score = (i^{th} value – mean)/SD. The muscular fitness z-score was calculated as the mean of the 2 standardized scores (handgrip strength and standing long jump).

Body composition

Children were scanned with dual-energy X-ray Absorptiometry (DXA) using the Hologic Discovery Wi (Hologic Series Discovery QDR, Bedford, MA, USA). The DXA equipment was calibrated at the start of each testing day by using a lumbar spine phantom as recommended by the manufacturer. All DXA scans and analyses were performed using the APEX software (version 4.0.2) following the same protocol by the same researcher. The positioning of the participants and the analyses of the results were undertaken following recommendations from the International Society of Clinical Densitometry (190). The total body scan was used to obtain fat mass, lean mass, and aBMD at the TBLH, arms and legs.

Statistical analysis

Descriptive characteristics of the participants are presented as mean ± standard deviation (SD) or percentages. All variables were checked for normality using visual check

of histograms, Q-Q and box plots. Interaction analyses were performed for sex and since no significant interactions were found (P≤0.28), analyses were performed for boys and girls together.

A partial correlation analysis controlling for sex and years from PHV was performed to examine the relationship between 25(OH)D, muscular fitness variables, TBLH lean mass and TBLH fat mass.

We carried out a mediation analysis controlling for sex, years from PHV, TBLH lean mass and season to test whether the association between 25(OH)D and aBMD outcomes was mediated by muscular fitness. These covariates were selected because of their well-known association with aBMD (170,205). The PROCESS macro version 3.1, model 4, with 10000 bias-corrected bootstrap samples and 95% confidence intervals was used for these analyses. In a nutshell, the mediation analysis is composed of ordinary least squared regression-based equations (paths) that allow us to answer the question of how a predictor transmits its effect (total effect) on an outcome being partitioned into direct (c' path) and indirect effect (a*b path). Most contemporary analysts focus on the indirect effect by stating 2 steps in establishing mediation (171): 1) show that the causal variable is correlated with the mediator (path a); 2) show that the mediator affects the outcome variable controlling for the predictor (path b). Thus, mediation is assessed by the indirect effect of the 25(OH)D (predictor) on aBMD (outcome) through muscular fitness (mediator). The total (c path), direct (c' path), and indirect effects (a*b paths) are presented in **Figure 1.** Indirect effects with confidence intervals not including zero were interpreted as statistically significant (171) regardless of the significance of the total effect (the effect of 25(OH)D on aBMD outcomes) and the direct effect (the effect on aBMD outcomes when both 25(OH)D and muscular fitness are included as independent

variables). The percentage of mediation (P_M) was calculated as '(indirect effect / total effect) × 100' to know how much of the total effect was explained by the mediation when the following assumptions were achieved: the total effect is larger than the indirect effect and of the same sign. All the analyses were performed using the IBM SPSS Statistics for Windows version 20.0 (Armonk, NY: IBM Corp), and the level of significance was set to $P < 0.05$.

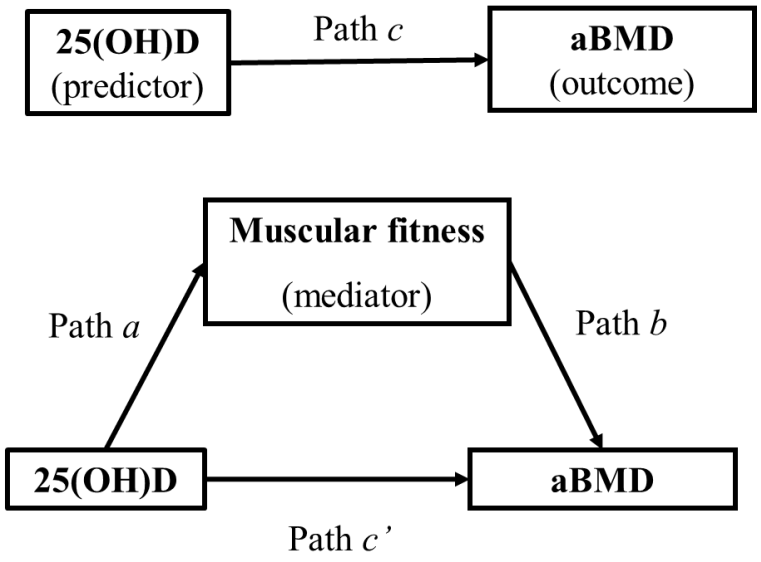

Figure 1. Causal diagram reflecting the simple mediation analyses. Path c shows the association between the predictor and the outcome. Arrows a × b show the natural indirect effect pathway, and c' shows the natural direct effect pathway.
aBMD areal bone mineral density

Results

Table 1 shows the raw descriptive characteristics of the participants at baseline (mean ± SD). Briefly, the mean age of the participants was 10.0±1.2 years and they were 2.4±0.9 years below PHV, overweight and obesity was evident in 28.4% and 71.6% of them, respectively; the mean 25(OH)D concentration was 31.5 nmol/L and only 6.2% of the children measured fell above the suggested cut-off of 50 nmol/L (227).

Table 1. Characteristics of the study sample by sex.

Variables	All (n = 81)	Boys (n = 53)	Girls (n = 28)
Age (years)	10.0±1.2	10.2±1.2	9.7±1.2
Years from PHV (years)	-2.4±0.9	-2.6±0.9	-1.8±1.1
Height (cm)	143.9±8.7	144.5±8.1	142.7±9.8
Body mass (kg)	54.8±10.7	55.8±10.7	53.1±10.8
TBLH fat mass (kg) [a]	21.9±5.8	22.1±5.9	21.5±5.8
TBLH lean mass (kg) [a]	26.6±5.2	27.3±4.9	25.5±5.3
BMI (kg · m^{-2})	26.3±3.4	26.5±3.4	25.9±3.3
Overweight (%)	28.4	26.4	32.1
Obesity (%)	71.6	73.6	67.9
Season			
Autumn (%)	91.4	90.6	92.9
Winter (%)	8.6	9.4	7.1
Vitamin D			
25(OH)D (nmol/L) [a] *	31.5±9.5	32.7±9.6	29.2±8.9
Deficiency (%)	46.9	43.4	53.6
Insufficiency (%)	46.9	49.1	42.9
Sufficiency (%)	6.2	7.5	3.6
Muscular fitness			
Muscular fitness z-score [b]	0.000±1.000	0.032±0.098	-0.061±1.037
Handgrip strength (kg)/body mass (kg) [a]	0.307±0.059	0.309±0.058	0.303±0.059
Standing long jump (cm) [a]	106.2±17.8	106.5±17.9	105.7±17.9
aBMD			
TBLH (g · m^{-2}) [a]	0.772±0.059	0.775±0.059	0.766±0.058
Arms (g · m^{-2}) [a]	0.607±0.041	0.613±0.041	0.596±0.040
Legs (g · m^{-2}) [a]	0.913±0.079	0.917±0.082	0.906±0.074

PHV peak height velocity; TBLH total body less head; BMI body mass index; 25(OH)D 25-hydroxyvitamin D; aBMD areal bone mineral density

[a] Values were Blom-transformed before analysis, but non-transformed values are presented

[b] Z-score mean computed from handgrip strength (kg/kg) and standing long jump (cm) tests

*Vitamin D status was defined as follows (227):

Sufficiency, > 50 nmol · L^{-1}; Insufficiency, 30-50 nmol/L^{-1}; Deficiency, <30 nmol/L^{-1}

Partial correlations between 25(OH)D, muscular fitness variables, TBLH fat mass and TBLH lean mass after adjustment for sex and years from PHV are presented in **Table 2.** 25(OH)D was positively correlated with muscular fitness z-score and handgrip strength (r= 0.28 and r= 0.29, respectively). Muscular fitness z-score was positively correlated with TBLH aBMD and arms aBMD (r= 0.24 and r= 0.35, respectively), whilst handgrip strength was positively correlated with arms aBMD (r= 0.32). Finally, standing long jump

was positively correlated with aBMD at TBLH, arms and legs (r= 0.27, r= 0.29 and r= 0.23, respectively).

Table 2. Partial coefficients of the independent variable with muscular fitness variables and aBMD outcomes adjusted for sex and years from PHV

	Muscular fitness z-score [b]	Handgrip strength/ body mass	Standing long jump	TBLH aBMD	Arms aBMD	Legs aBMD
25(OH)D	**0.275***	**0.285***	0.186	0.039	0.043	-0.011
Muscular fitness z-score [b]	-	**0.881****	**0.869****	**0.244***	**0.352***	0.182
Handgrip strength/ body mass		-	**0.540****	0.165	**0.320***	0.089
Standing long jump			-	**0.266***	**0.295***	**0.233***
TBLH aBMD				-	**0.764****	**0.894****
Arms aBMD					-	**0.577****

PHV peak height velocity; *25(OH)D* 25-hydroxyvitamin D; *TBLH* total body less head: *aBMD* areal bone mineral density
[b] Z-score mean computed from handgrip strength (kg/kg) and standing long jump (cm) tests
Boldface indicates statistical significance: *P < 0.050, **P < 0.001

Mediation analysis

Mediation analysis models are depicted in **Figure 2**. 25(OH)D was not significantly associated with any of the aBMD outcomes (c, total effect). Regarding path a, 25(OH)D was positively associated with muscular fitness z-score (**Figure 2A**, β = 0.257, P = 0.028) and handgrip strength (**Figure 2B**, β = 0.263, P = 0.024). In the path b, in all mediation models, muscular fitness was positively associated with TBLH aBMD (**Figure 2A**, β = 0.209, P = 0.004), arms aBMD (**Figure 2B**, β = 0.318, P < 0.001) and legs aBMD (**Figure 2C**, β = 0.189, P = 0.012). Finally, when 25(OH)D and muscular fitness were simultaneously included as independent variables (c', direct effect), aBMD outcomes were not predicted. There was a significant mediating effect of muscular fitness on the relationship of 25(OH)D with TBLH aBMD, arms aBMD and legs aBMD (P_M ranged from 49.6% to 68.3%).

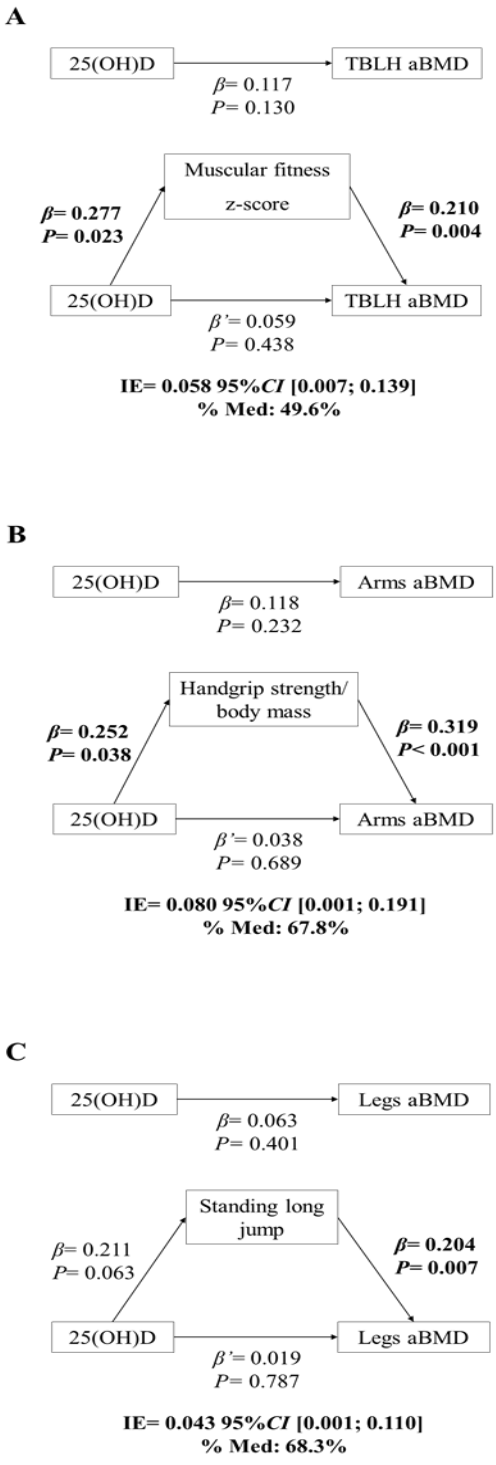

Figure 2. Simple mediation models of the relationship between 25(OH)D and aBMD outcomes using muscular fitness as a mediator, controlling for sex, years from PHV, TBLH lean mass and season. Muscular fitness z-score was used as mediator in panel A, handgrip strength/ body mass was used as mediator in panel B and standing long jump was used as mediator in panel C.
*****Z-score mean computed from handgrip strength (kg/kg) and standing long jump (cm) tests
PHV peak height velocity; *TBLH* total body less head; *25(OH)D* 25-hydroxyvitamin D; *aBMD* areal bone mineral density

Discussion

In the present study, we revealed a mediating effect of muscular fitness on the relationship between 25(OH)D levels and aBMD at the TBLH, arms and legs after controlling for sex, years from PHV, TBLH lean mass and season. To the best of our knowledge, this is the first study in children with overweight/obesity analyzing whether muscular fitness acts as mediator in the association between 25(OH)D and aBMD outcomes.

Our results show no significant association between 25(OH)D and aBMD outcomes after adjusting for sex, years from PHV, TBLH lean mass and season (path c, total effect). This finding agrees with Hauksson et al. (228) who found no significant association between 25(OH)D levels and bone mineral accrual in Icelandic children at ages 7 and 9. On the contrary, Pekkinen et al. (154) reported that 25(OH)D status was a key determinant of aBMD in children and adolescents. In this regard, 25(OH)D status has been highlighted as a significant predictor of peak bone mass in males but not in females during childhood (229). Likewise, non-significant associations between 25(OH)D and bone outcomes have been reported in American prepubertal girls after adjusting for potential cofounders (230) and in Finnish prepubertal girls after adjustment for maturation and BMI (161). This could be explained by the differences in sex hormone effects on bone since estrogens may counteract the effects of lower 25(OH)D levels in females, whereas in males this compensatory effect is absent (229). Nevertheless, we did not find sex interaction between 25(OH)D and aBMD outcomes, suggesting that these sex differences in hormonal effects on bone might not occur in prepubertal children with overweight/obesity since estradiol levels may be high in both boys and girls (231,232).

A few studies have assessed the effect of 25(OH)D in relation to muscular fitness in children (167,168,233). In addition, the present study does so, taking into account different ways of measuring muscular fitness in the upper and lower limbs. The results of the present investigation confirm a relationship between 25(OH)D levels and muscular fitness z-score, handgrip strength and standing long jump (path a). These results agree with Foo et al. (167) who observed that adolescent girls with sufficient 25(OH)D status performed significantly better in handgrip strength compared with those with deficient or severely deficient status. Moreover, our results partly concur with Ward et al. (168) who found a positive association between 25(OH)D levels and the performance in countermovement jump in British adolescent girls. Otherwise, a study carried out with children did not find any relationship between handgrip strength and 25(OH)D status (233).

In this study, muscular fitness z-score, handgrip strength and standing long jump were positively associated with TBLH aBMD, arms aBMD and legs aBMD, respectively (path b). Torres-Costoso et al. (144) reported a positive association between handgrip strength and aBMD outcomes in children aged 8-11 years, although a negative association between standing long jump and aBMD outcomes was found. The latter inverse association contrasts with our results. A possible explanation for these differences could be the different weight status of the participants included in both studies (BMI, 18.8±3.8 vs. 26.3±3.4). In addition, the fact that our results were adjusted for TBLH lean mass (but not in Torres-Costoso's study) could modify the direction of the association. Our findings agree with the literature and support the fact that bones adapt their resistance to the mechanical stimuli (i.e. body mass and muscle contractions) placed on them (127). Moreover, it should be noted that the performance in the standing

long jump test may be affected by the coordination skills (219), which might not be fully developed in 8-11-year-old children.

Our results show that the total effect of 25(OH)D on aBMD outcomes was mediated by muscular fitness z-score, handgrip strength and standing long jump (P_M ranged from 49.6% to 68.3%). Since the mediation analysis assumes that the predictor variable causes the mediator (225), muscular fitness may be an intermediate step in the causal pathway of 25(OH)D with aBMD. There is a consistent evidence regarding the bivariate association of muscular fitness with both 25(OH)D (234) and aBMD outcomes (235,236). Otherwise, the relationship between 25(OH)D and bone in children remains controversial (167,228). In addition, a recent study has reported that the association between muscular fitness and aBMD is fully mediated by lean mass, whose function appears to be affected by 25(OH)D levels (162). Together with our results, this evidence indicates that increasing 25(OH)D levels may increase muscular fitness and, ultimately, the aBMD. As an optimal bone mineral accrual is critical during childhood in order to prevent osteoporosis later in life (178), public health policies should start at early ages. Therefore, school-based interventions aiming at improving outdoor physical activity levels are justified among children to synthesize 25(OH)D and, ultimately, improve muscular fitness.

Strengths and limitations

The current study has several limitations that should be acknowledged. First, our cross-sectional design rules out the possibility of identifying cause-effect relationships. Thus, the reported findings need to be confirmed prospectively. Second, the number of participants with complete data in all studied variables is relatively small. Third, although

we did not find interaction by sex, our results need to be confirmed studying boys and girls separately. Finally, calcium intake was not available and therefore, we did not include it in the model as a cofounder (i.e. vitamin D interacts with calcium affecting bone health (149)).

The present study has also several strengths, such as the use of relevant sets of cofounders (i.e., sex, years from PHV, TBLH lean mass and season) that are crucial to analyse the association of 25(OH)D with bone outcomes in children. Furthermore, valid and reliable tests for assessing muscular fitness were chosen from the ALPHA-Fitness battery (226). Finally, we used DXA for assessing aBMD bone outcomes which is the gold standard for measuring bone outcomes and has been used worldwide in paediatric population (190).

Conclusions

Muscular fitness plays a key role in the relationship between 25(OH)D levels and aBMD at the TBLH and arms. Increasing 25(OH)D levels may improve muscular fitness and, ultimately, aBMD in children with overweight/obesity. Future longitudinal studies must be conducted in order to confirm these findings.

9. GENERAL DISCUSSION

The present Doctoral Thesis attempted to advance the existing knowledge on the bone-adiposity crosstalk through examining the relationship of inflammatory markers and adipokines with bone outcomes in children with overweight/obesity. Additionally, the role of muscular fitness modulating these associations was investigated for the first time. This Thesis also examined whether the vitamin D action on bone was mediated by muscular fitness in this population.

Section 1: Moderator role of muscular fitness in the association of inflammatory markers and adipokines with bone health.

The results from section 1 showed that some biochemical markers were associated with bone outcomes in children with overweight/obesity. First, we investigated the associations of 6 inflammatory markers (i.e., IL-1β, IL-6, TNF-α, EFG, VEGF, CRP) with TBLH BMC and LS BMC. We observed that IL-6 and VEGF levels were negatively associated with TBLH BMC, whereas TNF-α was negatively associated with LS BMC. Otherwise, IL-1β was positively associated with LS BMC. Second, we investigated the associations of leptin and adiponectin concentrations with bone outcomes (TBLH BMC, LS BMC, TBLH aBMD and LS aBMD). We found no association of adiponectin and leptin (borderline with LS BMC) with bone outcomes. According to Kawai et al. (85), inflammatory markers directly affect bone metabolism, whereas adipokines regulate central nervous system outflow from the sympathetic nervous system, and thus affect bone via b_2 adrenergic receptors. Of note, leptin levels have been reported to secrete some inflammatory markers (i.e., IL-1β, IL-6 and TNF-α) in children with overweight/obesity (237). With that in mind, the inclusion of inflammatory markers and adipokines in the same multiple regression analysis may be more illustrative, although

the small sample size ruled out the possibility of doing this. Likewise, the little scientific evidence on the relationship between adipokines and bone outcomes in children with overweight/obesity reinforces our election of separating both inflammatory markers and adipokines in two studies.

In addition, we tested whether optimal levels of objectively measured muscular fitness could modify these associations. Our first study observed that the negative association of VEGF and TNF-α with TBLH BMC and LS BMC disappeared in those children with higher levels of muscular fitness (mean score of upper- and lower-body muscular fitness). Similarly, the negative association between leptin and LS BMC found in those children with lower RM leg press also disappeared in those children higher RM leg press. The different ways in which muscular fitness was included on the analyses were due to the usage of PROCESS macro in the **study 2**. As previously described, this statistical model allowed us to clarify the specific RM leg press cut points in which the relationship between leptin and LS BMC changes (i.e., direction and/or significance).

Section 2: Mediator role of muscular fitness in the association between vitamin D and bone health

There is a considerable support in the extant literature for the vitamin D effect in muscular fitness (167–169) and bone health (154,155) in children and adolescents. However, there is a paucity of research investigating whether field-based muscular fitness can be the cornerstone to understand this relationship. Thereby, **study 3** highlighted that 25(OH)D levels were indirectly associated with aBMD at TBLH, arms and legs through relative muscular fitness (muscular fitness score, handgrip and SLJ) in children with overweight/obesity after adjusting for sex, years from PHV, lean mass and

season. Indeed, the percentage of mediation ranged from 49.6 to 68.3%. From a statistical point of view, although the associations between 25(OH)D and aBMD at TBLH, arms and legs were not significant, there existed correlation between predictor and mediator variables, and between mediator and outcome (i.e., aBMD at TBLH, arms). Therefore, although the mediation models were performed, the term mediation term should be avoided and use indirect associations instead.

With regard to section 1, previous studies reported vitamin D levels to be inversely correlated with IL-1, IL-6, TNF-α and CRP levels, NF-κB activity and cytokines levels from monocytes (238–240). Therefore, as previously mentioned, the inclusion of vitamin D in the same multiple regression analysis may be more illustrative, although the small sample size did not allow this inclusion. Moreover, unlike study 1 and study 2, this study used field-based muscular fitness tests. These tests have been reported to be valid and reliable to measure muscular fitness (226). Additionally, there existed correlation between objectively measured muscular fitness and field-based muscular fitness in our sample of children with overweight/obesity (r=0.424-0.640). Interestingly, we used the relative muscular fitness values (i.e., handgrip divided by body mass, SLJ and the mean score of both variables), since bigger children tend to be stronger but also have more fat mass, which implies the sequestration of vitamin D. This fact may hide the association between vitamin D and muscular fitness in this population, and thus we decided to use relative values instead of absolute.

Importantly, we only reported BMC parameters in the **study 1** and aBMD parameters in the **study 3**. For total body assessments, BMC has been reported to be the preferred method since its reproducibility and lack of areal density-related errors (241,242). However, Shepherd and colleagues showed that aBMD may be more precise measure

than BMC during child development (242,243). Given the International Society of Clinical densitometry recently stated that DXA reports should contain BMC and aBMD variables, we included both BMC and aBMD parameters in the **study 2** (the last manuscript drafted – to be *submitted)* of this Thesis. Furthermore, we included arms and legs aBMD in the **study 3** in order to check whether the vitamin D effects were site dependent according to muscular fitness variables.

Overall limitations and strengths of this Thesis

The present Doctoral Thesis has limitations that should be acknowledged. First, the cross-sectional design of the 3 studies rules out the possibility of identifying cause-effect relationships. Second, the number of participants with complete data in all studied variables was relatively small in each study. Third, the biochemical markers used in these 3 studies were obtained from plasma. Previous studies have used plasma or serum samples and therefore, comparisons may be affected. However, as shown in a recent study the correlations between plasma and serum measurements suggest that the differences in metabolite concentrations does not necessarily introduce a bias in cross-sectional studies (200). Fourth, lumbar spine DXA results were obtained from the whole-body scan instead of a region-specific scan, which is not informative **(study 1 and 2)** (215). We did not test the DXA error in estimating region-specific LS aBMD from whole-body scan, yet previous studies in adults have reported that this error ranged from 4.3% to 4.9% (244). Fifth, although we did not find interaction by sex in the association between 25(OH)D and aBMD, our results need to be confirmed by studying boys and girls separately (**study 3**).

Strengths of the present Doctoral Thesis include the use of DXA, which is the gold standard for measuring bone and have been used worldwide in the paediatric population (245). Besides, the accuracy of the objective methodology used for muscular fitness (**study 1 and 2**), and the use of valid and reliable tests for assessing muscular fitness in field-based conditions (**study 3**) (226). Finally, the use of relevant sets of cofounders such as sex, years from PHV and lean mass (**study 1 and 2**), and sex, years from PHV and TBLH lean mass and season (**study 3**).

Future research directions

- DXA-derived hip structural analyses and trabecular bone score should be incorporated in future studies aimed at examining the relationships of inflammatory markers and adipokines with bone health.
- 3D bone outcomes derived from peripheral quantitative computed tomography should be incorporated in future studies which will allow obtaining important information regarding changes in cortical and/or trabecular bone.
- Apart from these analyses, future longitudinal studies should investigate the relationships of inflammatory markers and adipokines with bone health from adolescence into adulthood in order to check whether the peak bone mass is compromised in this population.
- Future well-designed randomised controlled trials are needed to shed light on which exercise programmes influence bone health in children with overweight/obesity.
- In addition to inflammatory markers and adipokines, the inclusion of key (and novel) markers of bone metabolism, such as alkaline phosphatase, osteocalcin,

PINP, β-CTX, sclerostin and irisin will guarantee obtaining precise data on how exercise modulates muscle, fat and bone metabolism.

10. CONCLUDING REMARKS

CONCLUDING REMARKS

Overall conclusions

The findings of the present Doctoral Thesis provide new evidence regarding the association of several biochemical markers (i.e., inflammatory markers and adipokines) with bone outcomes, supporting that the link between obesity and bone health may be explained by some of them. Moreover, appropriate levels of muscular fitness may ameliorate some of the negative effects of these biochemical markers on bone, which reinforces the importance of enhancing optimal levels of muscular fitness early in life.

Besides, a novel pathway on how vitamin D acts on bone is provided in this Thesis. This Thesis highlighted that increasing 25(OH)D levels may improve muscular fitness and, ultimately, aBMD in children with overweight/obesity. Therefore, school-based interventions aimed at improving outdoor physical activity levels are justified among children to synthesize 25(OH)D and, ultimately, improve muscular fitness.

Specific conclusions

Study 1

IL-6 and VEGF were negatively associated with TBLH BMC, whereas TNF-α (negatively) and IL-1β (positively) were associated with LS BMC. Moreover, appropriate levels of muscular fitness (score including upper-body and lower-body muscular fitness) may attenuate the adverse effects of VEGF and TNF-α on TBLH BMC and LS BMC.

Study 2

Neither leptin nor adiponectin were associated with bone outcomes in children with overweight/obesity. Nevertheless, leptin levels were negatively associated with LS BMC in those children with low lower-body muscular fitness, whereas no association was found in those children with high lower-body muscular fitness in this population.

Study 3

Muscular fitness plays a key role in the relationship between 25(OH)D levels and aBMD at the TBLH, arms and legs.

11. ANNEXES

Short curriculum vitae

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ACADEMIC BACKGROUND

2018-2021	PhD student in Biomedicine. Research programme in Physical Activity and Sport, University of Granada, Granada, Spain.
2018-2020	Bachelor's degree in Primary Education (Specialization: Physical Education), Universidad Camilo José Cela, Madrid, Spain.
2016-2017	Master's degree in Education and Teaching in High School (Specialization: Physical Education), University of Granada, Granada, Spain.
2012-2016	Bachelor's degree in Sport Sciences, Faculty of Sport Sciences, University of Granada, Granada, Spain.

PREVIOUS FUNDING/POSITIONS

2021	Full time 6-month contract linked to the Towards Intelligent Health and Well Being. Network of Physical Activity Assessment, University of Granada, Granada, Spain.
2021	Full time 3-month contract linked to the iBoneFIT project (Ref: 100010434), University of Granada, Granada, Spain.
2020	Grant of the attendance to national or international congress 2020. University of Granada.
2020-2021	Full time 6-month contract linked to the Towards Intelligent Health and Well Being. Network of Physical Activity Assessment, University of Granada, Granada, Spain.
2019-2020	Full time 7-month contract linked to the iBoneFIT project (Ref: 100010434, LCF/BQ/PR19/11700007), University of Granada, Granada, Spain.
2017-2018	Full time 6-month contract linked to the ActiveBrains project (Ref: DEP2013-47540-R), the National Operational Programme for the implementation of the Youth Employment, University of Granada, Granada, Spain.
2016-2017	Research Assistantship Fellow. Centro Mixto UGR-MADOC (Ref: PIN UNGR15-DE-3312), University of Granada, Granada, Spain.

COURSES AND EXTRACURRICULAR ACTIVITIES

2020 Course: Advanced clinical trials' design and analyses. International Doctoral School, University of Granada.

2019 Workshop: Translating Exercise Science Research to Policy and Practice. Unidad Científica de Excelencia en Ejercicio y Salud (UCEES) and University California San Diego.

2019 Course: Software for the research development. International Doctoral School, University of Granada.

2019 Course: Basic clinical trials' design and analyses. International Doctoral School, University of Granada.

2018 Course: Data science, a practical approach in BIG DATA. Mediterranean Centre, University of Granada.

2018 Course: Statistical analyses on randomised controlled trials. Health and Social Research Centre, Universidad de Castilla La-Mancha.

2018 Course: Statistics in health sciences, an introductory approach to R. Faculty of Pharmacy, University of Granada.

2017 Certificate National Athletics School: National Athletics Coach. National Athletics Federation.

2017 Certificate in English: First Certificate in English ESOL International (level B2 English). University of Cambridge.

INTERNATIONAL RESEARCH INTERNSHIP

2021 Université Clermont Auvergne, Clermont-Ferrand, France.

Laboratoire de Adaptations Métaboliques à l'Exercice en conditions Physiologiques et Pathologiques (AME2P). Prof. Daniel Courteix

Laboratoire de Psychologie Sociale et Cognitive (LAPSCO). Prof. Frédéric Dutheil.

Duration: 3 months.

RESEARCH PROJECTS

2019-2022 The European Network for the Support of Development of Systems for Monitoring Physical Fitness of Children and Adolescents. Funded by: European Commission (400.000,00€). PI: Gregor Jurak

2019-2023 Investigating bone health in paediatric cancer survivors and its link with physiologic frailty: a multicentre study. Funded by: La Caixa Foundation (296.096,00€). PI: Luis Gracia Marco

2017 Effects of a moderate dose of beer on skiing technique, coordination, decision making, fatigue, postural stability and fitness in male recreational skiers. Funded by: Centro de Información Cerveza y Salud (29.870,00€). PI: Manuel Castillo Garzón

2015-2017 Effects of backpack load on biomechanical parameters, fatigue, body composition and fitness in army males. Funded by: Spanish Ministry of Economy and Competitiveness (377.297,15€). PI: José María Heredia

2014-2017 The ActiveBrains project. Funded by: Spanish Ministry of Economy and Competitiveness (120.000,00€). PI: Francisco B. Ortega

ACADEMIC/EDUCATIONAL ACTIVITY

2021 Invited lecturer in the Master's degree in Physical Activity and Sport Research, University of Granada, Granada, Spain.

2021 Seminar in mediation analyses in physical activity and bone outcomes. Université Clermont-Auvergne, Clermont-Ferrand, France.

2020 Seminar in moderation analyses with macro PROCESS for SPSS. University of Granada, Granada, Spain.

2020 Invited lecturer in the Master's degree in Physical Activity and Sport Research, University of Granada, Granada, Spain.

2020 Invited lecturer in the European Research Night, University of Granada, Granada, Spain.

2019 Invited lecturer in the European Research Night, University of Granada, Granada, Spain.

2017 Invited lecturer in the Master's degree in Education and Teaching in High School (Specialization: Physical Education), University of Granada, Granada, Spain.

ARTICLES INCLUDED IN THE THESIS

PUBLISHED PAPERS

- **Gil-cosano JJ**, Gracia-marco L, Ubago-guisado E, Migueles JH, Mora-Gonzalez J, Escolano-Margarit MV, Gómez-Vida J, Maldonado J, Ortega FB. Muscular Fitness Mediates the Association between 25-Hydroxyvitamin D and Areal Bone Mineral Density in children with overweight/obesity. Nutrients. 2019;11(11):2760.
- **Gil-Cosano JJ**, Gracia-Marco L, Ubago-Guisado E, Labayen I, Adelantado-Renau M, Cadenas-Sanchez C, Mora-Gonzalez J, Plaza-Florido A, Aguilera CM, Gómez-Vida J, Maldonado J, Jürimäe J, Ortega FB. Inflammatory markers and bone mass in children with overweight/obesity: the role of muscular fitness. Pediatr Res. 2020;87(1):42–7.

SUBMITTED ARTICLES

- **Gil-Cosano JJ**, Gracia-marco L, Ubago-guisado E, Migueles JH, Courteix D, Labayen I, Plaza-Florido A, Molina-García P, Dutheil F, Ortega FB. Adipokines and bone outcomes in children with overweight/obesity: The moderator role of muscular fitness. Pediatr Res.

ARTICLES IN PREPARATION NOT INCLUDED IN THE THESIS

- **Gil-Cosano JJ**, Gracia-Marco L, Migueles JH, Ortega FB et al. Effects of a 4.5-months exercise programme on bone health in children with overweight/obesity.
- **Gil-Cosano JJ**, Courteix D, Gracia-Marco L, Ortega FB, Dutheil F et al. Visceral adipose tissue and bone turnover markers in adults: the mediator role of tumour necrosis factor alpha and vascular endothelial growth factor.

OTHER ARTICLES AS FIRST AUTHOR OR COAUTHOR

1. Latorre-Román PÁ, Soto-Hermoso VM, García-Pinillos F, **Gil-Cosano JJ**, Robles-Fuentes A, Muñoz-Jiménez M, Molina-Molina A. Fatigue's effects on spatiotemporal parameters and foot-strike patterns during a half marathon. Rev Int Med Cienc Act Fis Deporte. 2021. *In press*
2. Gracia-Marco L, Gonzalez-Salvatierra S, Garcia-Martin A, Ubago-Guisado E, Garcia-Fontana B, **Gil-Cosano JJ**, Muñoz-Torres M. 3D DXA Hip Differences in Patients with Acromegaly or Adult Growth Hormone Deficiency. J Clin Med. 2021;10(4):657.
3. **Gil-Cosano JJ**, Ubago-Guisado E, Sánchez MJ, Ortega-Acosta MJ, Mateos ME, Benito-Bernal AI, Llorente-Cantarero FJ, Ortega FB, Ruiz JR, Labayen I, Martínez-Vizcaíno V, Vlachopoulos D, Arroyo-Morales M, Muñoz-Torres M, Pascual-Gázquez JF, Vicho-González MC, Gracia-Marco L. The effect of an online exercise programme on bone health in paediatric cancer survivors (iBoneFIT): Study protocol of a multi-centre randomized controlled trial. BMC Public Health. 2020;20(1):1–13.
4. Molina-Garcia P, Mora-Gonzalez J, Migueles JH, Rodriguez-Ayllon M, Esteban-Cornejo I, Cadenas-Sanchez C, Plaza-Florido A, **Gil-Cosano JJ**, Pelaez-Perez MA, García-Delgado G, Vanrenterghem J, Ortega FB. Effects of Exercise on Body Posture, Functional Movement, and Physical Fitness in Children With Overweight/Obesity. J strength Cond Res. 2020;34(8):2146–55.
5. Migueles JH, Cadenas-Sanchez C, Rowlands AV, Henriksson P, Shiroma EJ, Acosta FM, Rodriguez-Ayllon M, Esteban-Cornejo I, Plaza-Florido A, **Gil-Cosano JJ**, Ekelund U, Van Hees VT, Ortega FB. Comparability of accelerometer signal aggregation metrics across placements and dominant wrist cut points for the assessment of physical activity in adults. Sci Rep. 2019;9(1):1–12.
6. Adelantado-Renau M, Esteban-Cornejo I, Rodriguez-Ayllon M, Cadenas-Sanchez C, **Gil-Cosano JJ**, Mora-Gonzalez J, Solis-Urra P, Verdejo-Román J, Aguilera CM, Escolano-Margarit MV, Verdejo-Garcia A, Catena A, Moliner-Urdiales D, Ortega FB. Inflammatory biomarkers and brain health indicators in children with overweight and obesity: The ActiveBrains project. Brain Behav Immun. 2019;81:588–97.
7. **Gil-Cosano JJ**, Orantes-Gonzalez E, Heredia-Jimenez J. Effect of carrying different military equipment during a fatigue test on shooting performance. Eur J Sport Sci. 2019;19(2):186–91.

ACCEPTED CONGRESS COMMUNICATIONS AS FIRST AUTHOR

1. **Gil-Cosano JJ**, Gracia-Marco L, Ubago-Guisado E, Migueles JH, Plaza-Florido A, Maldonado J, Escolano-Margarit MV, Gómez-Vida J, Ortega FB. [The association between adipokines and bone mass in children with overweight/obesity: the role of muscular fitness]. October 28-30th, 2020
2. **Gil-Cosano JJ**, Gracia-Marco L, Migueles JH, Plaza-Florido A, Maldonado J, Escolano-Margarit MV, Gómez-Vida J, Ortega FB. [Vigorous physical activity and bone mass in children with overweight/obesity: the mediating role of cardiorespiratory fitness and leptin]. International Workshop: A focus on statistical methods to analyse accelerometer-measured physical activity. Granada, Spain. October 21st, 2019.
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REVIEWER IN JCR JOURNALS

- BMC Public Health
- Nutrients
- Acta Paediatrica
- Frontiers in Psychology

OTHER

2020 Book chapter: Arias-Tellez MJ, **Gil-Cosano JJ**, de Lucena Martins C. Lácteos, actividad física y su efecto sinérgico sobre salud ósea y muscular. Editor: Rodrigo Valenzuela. *Lácteos: Nutrición y Salud.* ISBN: 978-956-8765-11-8

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12. ACKNOWLEDGEMENTS/ AGRADECIMIENTOS

Me gustaría comenzar esta sección aludiendo a la dedicatoria inicial de este libro: *"A los Gigantes, sin los cuales esta Tesis no existiría"*. Y es que, citando a Isaac Newton (1643-1727), *"Si he visto más lejos es porque estoy sentado sobre hombros de gigantes"*. En otras palabras, los resultados de la presente Tesis Doctoral son gracias al apoyo y generosidad de personas que han hecho de estos tres últimos años un camino más cómodo y lleno de aprendizaje. A continuación, os presento a mis gigantes: directores, equipo de trabajo, amigos y familia.

Gigante 1 – Directores

Antes de tomar la decisión de realizar la Tesis Doctoral, vi una ponencia TED ("*Como hacer un doctorado: lo que no te han contado, ni te contarán", José Luis Arroyo*) que mencionaba la importancia de elegir un buen director de Tesis. En concreto, el ponente decía que un buen director debía reunir dos cualidades: competitividad y competencia. Entendiéndose por competitividad los índices de impacto científico, proyectos científicos y redes de trabajo; y por competencia la capacidad o motivación del director para guiar el aprendizaje del doctorando hasta convertirse en doctor. En otras palabras, ser un buen líder.

Fran, desde mis inicios en ActiveBrains pude captar esa competencia. Tu trato cercano y asertivo con los integrantes del equipo, sumado al entusiasmo por la ciencia y tu capacidad para transmitir conocimientos complejos de una manera sencilla, influyeron exponencialmente mi interés hacia la investigación. Años después pude comprobar que tu nivel de competitividad estaba a la altura del nivel de competencia, por lo que acabar trabajando bajo tu supervisión ha sido un privilegio desde el punto de

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Gigante 2 – Equipo de trabajo

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