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ORIGINAL ARTICLE

The role of pro/anti-inflammatory adipokines on bone metabolism in NAFLD obese adolescents: effects of long-term interdisciplinary therapy

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Abstract To investigate the role of pro- and anti-inflammatory adipokines in the bone metabolism of non-alcoholic fatty liver disease (NAFLD) obese adolescents as well as the effects of long-term interdisciplinary therapy on metabolic-related risk factors. Forty post-puberty obese adolescents were randomly assigned into two groups: (1) NAFLD group and (2) non-NAFLD group (diagnosis by ultrasonography) and submitted to a weight loss therapy. Body composition was analyzed by air displacement plethysmography, bone mineral density (BMD) and content by dual-energy X-ray absorptiometry, blood samples were collected to measure lipid profile, hepatic enzymes, and adipokines. Leptin and adiponectin concentrations were measured by ELISA. A decrease in total body mass, BMI, body fat, visceral and subcutaneous fat, insulin concentration, HOMA-IR, total cholesterol and an increase in lean body mass were observed in both groups after therapy. It was found positive correlation between the Δ BMD and the Δ fat mass (%) ($r = 0.31$, $P = 0.01$) and negative correlations between Δ BMC with Δ HOMA-IR ($r = -0.34$, $P = 0.02$) and Δ HOMA-IR with Δ leptin ($r = -0.34$, $P = 0.02$). In addition, increased levels of adiponectin and reduction in leptin concentrations were observed in NAFLD group. In the simple regression analysis, the HOMA-IR was an independent predictor changes in BMC in total obese adolescents and in the non-NAFLD group. One year of interdisciplinary weight loss therapy for obese adolescents with or without NAFLD, could regulate bone mineral metabolism as result of an increased BMC and improved inflammatory state.

Keywords Obesity · Bone mass · Leptin · Adiponectin · NAFLD

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Introduction

Peak bone mass is often achieved by early adulthood, and adolescence is considered to be a critical period for the accumulation of a significant quantity of bone mass. In this way, any disease or condition that reduces bone mineral accrual during this period may lead to a suboptimal peak bone mass, with presumably greater risk of fractures later in life as well as significantly compromised bone health [1].

The factors that are associated with the acquisition of bone mass can be divided into genetic and environmental

categories. The variance in bone mineral density (BMD) is influenced by both intrinsic and extrinsic environmental factors such as sex, weight, hormonal exposures, physical activity, sunlight exposure, diet, and disease state. The promotion of pediatric bone health in children through healthy eating habits, increased exercise, and reduced sedentary activities, should be a priority for all pediatricians. This lifestyle therapy will also help fight the alarming rise in childhood obesity and the increasingly sedentary nature of children [2].

However, the effect of childhood obesity on bone mineral content and density is controversial. There has recently been a growing concern that childhood obesity may negatively affect bone development, as a link between childhood obesity and skeletal fractures has been observed [3].

During childhood and adolescence, bone mineral accretion results in sex- and maturation-specific increases in cortical dimension and trabecular density [4]. These periods are crucial for bone growth, as only half of bone mass is achieved in adulthood. Indeed, peak bone mass may provide significant protection against osteoporotic fracture risk later in life, which reinforces the importance of childhood detection of low bone mass and intervention programs at an early age [5].

Despite the epidemic of adolescent obesity, the effect of obesity and hormones on bone mineral accrual during growth is poorly understood. Studies using dual-energy X-ray (DXA) to examine the effect of obesity on bone mass in children and adolescents have yielded conflicting results. However, it has been suggested that proinflammatory cytokines and low-grade systemic inflammation activate bone resorption and may lead to reduced BMD [6].

A previous study of obese adolescents found that leptin, insulin, and HOMA-IR levels were inversely associated with BMD and played a significant direct role in bone metabolism [7]. Indeed, another study supported the concept that insulin resistance, a lower high-density lipoprotein (HDL) cholesterol level and visceral adiposity may adversely influence adolescent bone mass [1]. These data suggest that altered metabolic parameters, such as insulin resistance, can exert influence on bone mineral content.

It was showed a relationship between insulin resistance and non-alcoholic fatty liver disease (NAFLD) development in obese adolescents and also found that expansion of visceral fat was a determinant for increased non-alcoholic fatty liver disease prevalence [8–11]. Although the disease mechanisms responsible for pediatric NAFLD need to be further investigated, insulin resistance and visceral fat accumulation appear to be critical for this disease [12]. This metabolic disequilibrium leads to the accumulation of fatty acid in the liver causing an increase in β-oxidation and oxidative stress that affect hepatic secretion of proinflammatory cytokines [13].

Moreover, a reduction in BMC in NAFLD obese adolescents was recently demonstrated. However, it was unclear whether this parameter was improved by weight loss therapy as part of a long-term therapy, and the role of adipokines in this inflammatory state was not identified [14]. In this way, diseases and conditions with insulin resistance as the central pathogenic factor, such as NAFLD, could be related to a worsened bone mineral metabolism.

Furthermore, experimental evidence has indicated that white adipose tissue-derived adipokines also contribute to the pathogenesis of NAFLD, and, hypoadiponectinemia has been implicated in the development of insulin resistance, hepatic fat accumulation, and abnormal liver enzymes [15, 16]. Adiponectin acts as an insulin sensitizer promoting anti-inflammatory effects. However, the serum levels of adiponectin have been shown to be decreased in obesity and NAFLD and are negatively associated with HOMA-IR [17, 18].

Hyperleptinemia is commonly observed in obese humans and animals being recently suggested its pro-inflammatory effect [19–21]. Inversely, as cited above, a decrease in the adiponectin concentration was observed in obese adolescents and adults with NAFLD. Moreover, it was suggested that the ratio of leptin/adiponectin is a great indication of inflammatory state in human [22]. However, the potential mechanisms behind the diminished adiponectinemia and hyperleptinemia as well as the relationship of these conditions to inflammation remain to be investigated in obese adolescents [15, 17].

We hypothesized that adipokines such as leptin and adiponectin, as well as other metabolic parameters may significantly influence the bone health in the pediatric obese population. Indeed, early strategies to attenuate the inflammatory state noted in NAFLD obese group must be applied to promote bone development during this critical period of adolescence. In this way, the first aim of this study was to assess the effects of long-term interdisciplinary therapy on metabolic-related risk factors of NAFLD obese adolescents; and second, was to evaluate the role of pro- and anti-inflammatory adipokines in bone metabolism in this population.

Study protocol

Forty post-puberty obese adolescents (NAFLD group: 18; non-NAFLD group: 22) with a mean age of 17 $\pm$ 1.7 years (15–19) were enrolled in this study. Inclusion criteria were Tanner stage 5 [23], primary obesity (grade I and II), BMI >30 kg/m^2 (BMI >95th percentile of the CDC reference growth charts) and sedentary lifestyle for at least 6 months. Exclusion criteria were the use of birth control pills,

cortisone, anti-epileptic drugs, history of renal disease, alcohol intake, smoking, secondary obesity due endocrine disorders, history of fractures and fixed assets, long-term supplementation of calcium and or other drugs that can affect bone metabolism. There were no obese adolescents with diagnoses of diabetes mellitus.

The main reasons for dropping out in our study were financial and family problems, followed by school and job opportunities. No sex differences were observed in adherence rates. The patients were paired according to age, body fat mass percentage, and lean body mass percentage. The study was conducted with the principles of the Declaration of Helsinki and was approved by the ethics committee on research at the Universidade Federal de São Paulo—UNIFESP with the number (#0135/04), Clinical Trials.gov NCT01358773. All procedures were clear to those responsible for the volunteers and it was obtained consent for research. All evaluations were performed at two different times (baseline: beginning and end of therapy: after 1 year of therapy).

Anthropometric measures

Weight was measured by plethysmography scale (BODPOD equipment), where patients wore minimum clothing possible and height was measured using a stadiometer (Sanny—model ES 2030). After obtaining the data was calculated using the body mass index (BMI) by dividing the weight by height squared (kg/m^2). Body composition, including body fat (percentage and kilograms) and lean mass (percentage and kilograms), was obtained through BODPOD.

Serum analysis

Blood samples were collected at the outpatient clinic at approximately 8:00 A.M. after an overnight fast, and the fasting blood glucose (FBG). The concentrations of leptin and adiponectin were measured using commercial immunoassay kits eBioscience (San Diego, CA, USA) and R&D Systems (Minneapolis, MN, USA) according to manufacturers' manual. The reference values for leptin have been previously described by Gutin et al. [24]. For the leptin hormone, the following values were adopted: males, between 1 and 20 ng/ml and females, between 4.9 and 24 ng/ml.

Insulin resistance was assessed by the homeostasis model assessment insulin resistance index (HOMA-IR). HOMA-IR was calculated using the FBG and the immunoreactive insulin (I): [FBG (mg/dl) $\times$ I (mU/l)]/405. QUICKI (quantitative insulin sensitivity check index) was calculated as 1/(log I + log FBG). Total cholesterol, triglyceride (TG), HDL, low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), and the hepatic transaminases (alanine aminotransferase [ALT], aspartate aminotransferase [AST], and γ-glutamyl transferase [GGT]) were analyzed using a commercial kit (CELM, Barueri, Brazil). The reference values were: glucose (60–110 mg/dl), insulin (<20 μU/ml), HOMA-IR (<2.0), QUICKI (>0.339), total cholesterol (<170 mg/dl), TG (33–129 mg/dl), HDL cholesterol (>38 mg/dl), LDL cholesterol (<130 mg/dl), VLDL cholesterol (10–50 mg/dl), AST (10–40 U/l), ALT (10–35 U/l), and GGT (17–30 U/l) as previously described by Schwimmer et al. [25].

Bone mineral density and bone mineral content

A whole-body DXA absorptiometry scan was performed to determine the whole-body BMD (units: g/cm^2) and BMC (units: grams) using a HOLOGIC QDR4200 system (Hologic, Bedford, MA, USA). The whole-body scan required the subjects to be placed in a supine position with their arms and legs positioned according to the manufacturer's specifications. Quality control was performed daily using a phantom, and the measurements were maintained within the manufactures standards of $\leq$1% [26].

Hepatic steatosis, visceral and subcutaneous adiposity measurements

The abdominal ultrasonography procedures and the measurements of visceral and subcutaneous fat tissue and fatty liver were performed by the same physician, who was blinded to the subject assignment groups at the baseline time-point and following 1 year therapy. This physician was a specialist in imaging diagnostics. A 3.5-MHz multifrequency transducer (broad band) was used to reduce the risk of misclassification. The intra-examination coefficient of the variation for ultrasound (US) was 0.8%. US measurements of intra-abdominal (visceral) and subcutaneous fat were obtained. US-determined subcutaneous fat was defined as the distance between the skin and external face of the rectus abdominal muscle, and visceral fat was defined as the distance between the internal face of the same muscle and the anterior wall of the aorta. The cut-off points for the definition of visceral obesity by ultrasonography were based on the previous methodological descriptions made by Ribeiro-Filho et al. [27]. Steatosis evaluation was performed by abdominal ultrasonography. The definition of ultrasonic fatty liver was based on previously reported diagnostic criteria and detected liver steatosis was classified as grade 1 (liver attenuation slightly less than spleen); grade 2 (more pronounced difference

between liver and spleen and intrahepatic vessels not seen or slightly higher attenuation than liver); grade 3 (markedly reduced liver attenuation with sharp contrast between liver and intrahepatic vessels) [28, 29]. In this study, the group with NAFLD presented some liver steatosis grade diagnosed by U.S.

Clinical therapy

All obese adolescents visited the endocrinologist with their parents once each month. In all of these visits, the entire GEO team (Study Group of Obesity) was also present. The doctor monitored and evaluated all clinical exams of adolescents and treated health problems during therapy. The medical follow-up included the initial medical history and a physical examination of blood pressure, cardiac frequency and body weight, and the adolescents were checked for their adherence to all interdisciplinary therapies. The team discussed with the adolescents and their parents some possible changes in lifestyle to promote their health.

Dietary therapy

Energy intake was set at the level that was recommended by the dietary reference intake for subjects with low levels of physical activity of the same age and gender who follow a balanced diet [30]. All patients received a hypocaloric diet, which was adjusted by the nutritionist according to individual characteristics and habits. No drugs or antioxidants were recommended. Once a week, the adolescents had dietetic lessons [information was provided regarding the food pyramid, diet record assessment, weight loss diets and fad diets, food labels, dietetics, fat-free and low-calorie foods, fats (kinds, sources and substitutes), fast-food calories and nutritional composition, good nutritional choices for special occasions, healthy sandwiches, shakes and products to promote weight loss, functional foods and decisions on food choices]. All adolescents received individual nutritional consultation during the therapy and an educational program was proposed to improve the control of NAFLD, like previously described by Dâmaso et al. [31].

At the beginning of the study and after the 1 year therapy, a 3-day dietary record was collected. Portions were measured in terms of familiar volumes and sizes, and a dietician taught the parents and the adolescents how to record food consumption. The dietician the transferred to a computer, and the nutrient composition was analyzed using a software program that was developed at the Universidade Federal de São Paulo (Nutwin version 1.5 for Windows, 2002) and that used data from western diet and local food tables. In addition, parents were encouraged to contact the dietician if they needed extra information.

Physical exercise therapy

Aerobic plus resistance training (AT + RT)

During the 1-year therapy period, the adolescents followed a combined exercise training therapy. The protocol was performed three times per week for 1 year and included 30 min of aerobic training plus 30 min of resistance training per session. The subjects were instructed to reverse the order of the exercises (aerobic and resistance) at each training session. The aerobic training consisted of running on a motor-driven treadmill (Life Fitness—model TR 9700HR) at a cardiac frequency intensity representing the ventilatory threshold I (±4 bpm), which was determined by the results of an initial oxygen uptake test for aerobic exercises. The exercise therapy was based on the guidelines from the American College of Sports Medicine [32]. Resistance training was also designed based on ACSM recommendations [33].

Psychological therapy

Diagnoses of psychological problems most commonly associated with obesity such as depression, body image disturbance, anxiety and lower self-esteem, were assessed through validated questionnaires. During long-term intervention, adolescents were followed up weekly in the 1 year therapy support group, and if necessary, individual psychological therapy was recommended when behavioral alterations were found.

During psychological therapy, all adolescents completed the Portuguese versions of the BES [34] to verify the symptoms of binge eating and BITE to verify Bulimia symptoms, including the purgative subtype [35]. These tests were based on DSM-IV criteria and were validated for obese individuals, including obese adolescents, submitted to weight loss treatment [36–38]. It is relevant to note that the tests were applied only to identify the symptoms and severity of these disorders and not with the purpose of offering a diagnosis, because clinical interviews are necessary for confirmation (data not showed) (Fig. 1).

Statistical analysis

The data are presented as the means standard deviation, and P values <0.05 were considered statistically significant. Distributional assumptions were verified using the Kolmogorov–Smirnov test, and non-parametric methods were performed where appropriate. Adipokines levels were analyzed using parametric tests and were expressed as means ± SD unless otherwise stated. Comparisons

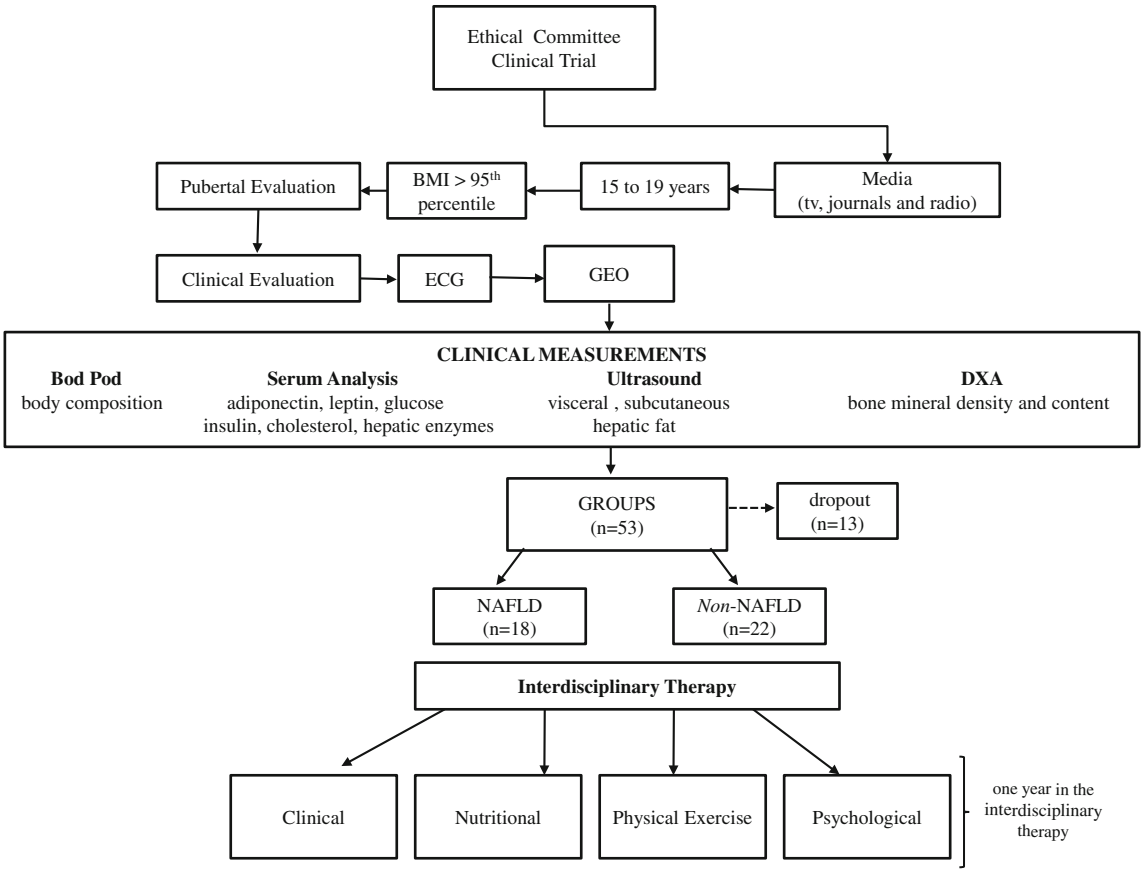

Fig. 1 Description of the methodology adopted to develop the study

between the baseline measurements and the measurements made after the weight loss therapy were made using the paired student's *t* test or the Wilcoxon signed-rank test for non-parametric variables. Comparisons between groups were performed using the independent student's *t* test or the Mann–Whitney test (non-parametric variables). Correlations analyses between fat mass, insulin level, and HOMA-IR value for BMD and BMC were performed using Pearson's correlation test. A stepwise simple linear regression using the BMC as dependent factor was performed. The delta variation (Δ) was used for the statistical analysis obtained from the difference between the baseline and final values for each variable. All analyses were computed using the STATISTICA version 6 for Windows.

Results

NAFLD group

After the weight loss therapy, there were significant decreased in BM, BMI, body fat mass (% and kg), visceral and subcutaneous fat, insulin concentration, HOMA-IR, total cholesterol, Lep/Adipo ratio, ALT, and GGT. An increase in lean body mass (% and kg) and BMC (g) was also observed. The mean weight loss observed in this group was 11 ± 7.2 (kg). Based on the adipokine data, we verified an increase in the adiponectin and a reduction in the leptin concentrations. We did not find significant change in AST (Tables 1, 2). Indeed the prevalence of NAFLD was decreased from 100 to 33% after therapy.

From the correlation analysis, a positive association was made between HOMA-IR and leptin concentration. Indeed, the Δ BMD was positively related to the Δ value for total cholesterol (Table 3).

Non-NAFLD group

After the 1 year of weight loss therapy, we observed significant reduction in BM, BMI, body fat mass (% and kg), visceral and subcutaneous fat, insulin, total cholesterol and LDL concentrations and HOMA-IR, which was similar to the trend observed for the NAFLD group. The mean weight loss observed in this group was 9 ± 8.3 (kg). Moreover, long-term (1 year) treatment have shown to promote a significant increase in HDL concentration, lean body mass (kg and %), and BMC (Tables 1, 2). We did not showed significant changes in the all analyzed hepatic enzymes.

Table 1 Effects of interdisciplinary therapy: body composition

Variables	NAFLD ($n = 18$)			Non-NAFLD ($n = 22$)		
	Baseline	1 Year	Δ Value	Baseline	1 Year	Δ Value
Total body mass (kg)	113.8 ± 16.7	102.5 ± 18.2†	−11.3 ± 7.2	98.0 ± 12.5*	88.9 ± 13.7**,†	−9.0 ± 8.3
Height (m)	1.65 ± 0.1	1.68 ± 0.1	−3.32 ± 0.1	1.68 ± 0.1	1.69 ± 0.1	−3.38 ± 0.1
BMI (kg/m^2)	39.9 ± 5.1	35.8 ± 5.9†	−4.1 ± 2.5	35.8 ± 5.3*	32.2 ± 5.0†	−3.5 ± 3.2
Fat body mass (%)	46.5 ± 6.5	37.8 ± 9.1†	−84.2 ± 15.0	45.5 ± 7.2	36.0 ± 8.6†	−81.5 ± 15.2
Lean body mass (%)	53.5 ± 6.5	62.2 ± 9.1†	8.7 ± 5.0	54.5 ± 7.2	64.0 ± 8.6†	9.5 ± 4.5
Fat body mass (kg)	53.4 ± 13.0	39.5 ± 14.2†	−13.8 ± 6.5	45.0 ± 11.3*	33.0 ± 11.9†	−12.0 ± 7.4
Lean body mass (kg)	60.5 ± 8.3	63.3 ± 9.3†	2.8 ± 5.8	53.1 ± 7.2*	56.2 ± 7.0**,†	3.0 ± 3.5
Total BMD (g/cm^2)	1.2 ± 0.1	1.2 ± 0.1	0.0 ± 0.0	1.2 ± 0.1	1.2 ± 0.1	0.0 ± 0.0
Total BMC (g)	2767.0 ± 498.3	2942.0 ± 549.9†	175.0 ± 210.2	2835.6 ± 371.2*	2945.2 ± 419.7†	109.7 ± 189.9
Total BMD (*Z* score)	1.5 ± 1.0	1.6 ± 1.1	0.2 ± 0.5	1.6 ± 1.0	1.7 ± 1.1	0.1 ± 0.3
Visceral fat (cm)	5.7 ± 1.5	3.8 ± 1.5†	−2.0 ± 1.3	4.1 ± 1.2*	2.5 ± 1.1**,†	−1.6 ± 1.2
Subcutaneous fat (cm)	4.0 ± 1.1	3.5 ± 0.9†	−0.5 ± 0.7	3.8 ± 0.8	3.4 ± 0.7†	−0.4 ± 0.8

NAFLD non-alcoholic fatty liver disease, *BMI* body mass index, *BMD* bone mineral density, *BMC* bone mineral content

* Statistical differences in baseline values between NAFLD and non-NAFLD group ($P < 0.05$)

** Statistical differences in year values between NAFLD and non-NAFLD group ($P < 0.05$)

† Statistical differences in basal vs year (therapy effect) in the same group ($P < 0.05$)

Comparison between the groups

At the baseline condition, is important to note that the NAFLD group presented greater values for the total body mass (kg), BMI, fat and lean mass (kg), visceral fat, insulin level, HOMA-IR, ALT and GGT and lower BMC. After long-term treatment has shown significantly greater values for total body mass, lean mass (kg), visceral fat, insulin, and HOMA-IR than non-NAFLD group (Tables 1, 2). However, improvement in BMC, subcutaneous fat, HOMA-IR, adiponectin, and lean body mass (kg) were observed in both groups after 1 year of therapy. We did not find statistical difference in the BMD, glucose and cholesterol in both analyzed groups.

From the analysis of the magnitude of the changes in each variable using the Δ calculation, there was found to be a major variation in the levels of insulin, HOMA-IR, and ALT and a minor variation in the concentration of HDL in the NAFLD group (Table 3).

There was a positive correlation between the Δ BMD and the Δ value for fat mass percentage ($r = 0.31$, $P = 0.01$) for the entire group (Table 3). On the other hand, the Δ BMC was negatively correlated with the Δ HOMA-IR ($r = -0.34$, $P = 0.02$) (Table 3). Moreover, a negative association was noted between changes in HOMA-IR and changes in leptin concentration ($r = -0.34$, $P = 0.02$) (Table 3). As shown in Fig. 2a and b, it could be suggested that the HOMA-IR was found to be independent predictor for changes in BMC in total obese adolescents and in the non-NAFLD group. BMC was the dependent variable used for the simple regression analysis.

Discussion

In this study, a 1-year interdisciplinary therapy period was able to promote changes in the metabolic profile of obese adolescents (*NAFLD and non-NAFLD*), including a decrease in the BM, BMI, body fat, visceral and subcutaneous fat, insulin concentration, HOMA-IR, total cholesterol and an increased in lean mass (% and kg). This result is important, since the improvement of these variables will decrease the risk of these individuals to develop other comorbidities in adulthood, such as metabolic syndrome, hypertension, and coronary heart disease [13]. According to Table 1, although the mean of weight loss for total patients was 10 kg we could observe that the maximum in the NAFLD group was 18.5 kg; and in the non-NALFD group was 17.3 kg, promoting an improvement in metabolic and hormonal profiles. These results corroborate to the importance of this kind of intervention to ameliorate health of obese adolescents.

At the baseline condition, NAFLD group presented statistically lower values of BMC, however, after 1 year of interdisciplinary therapy, there was an increase of BMC, reaching similar values of non-NAFLD group. This result can be partially associated with the beneficial effects of exercise with others therapy, improving some markers of

Table 2 Effects of interdisciplinary therapy: metabolic profile

Variables	NAFLD ($n = 18$)			Non-NAFLD ($n = 22$)		
	Baseline	1 Year	Δ Value	Baseline	1 Year	Δ Value
Glucose (μU/ml)	5.1 ± 0.5	5.0 ± 0.4	−0.1 ± 0.4	4.9 ± 0.3	4.8 ± 0.3	−0.1 ± 0.5
Insulin (μU/ml)	25.5 ± 16.0	15.0 ± 10.5†	−10.5 ± 9.9	14.3 ± 5.7*	9.7 ± 4.5**,†	−4.6 ± 4.8***
HOMA-IR	6.0 ± 4.2	3.5 ± 2.7†	−2.5 ± 2.5	3.1 ± 1.3*	2.1 ± 1.0**,†	−1.0 ± 1.1***
QUICK	0.49 ± 0.1	0.57 ± 0.1†	0.06 ± 0.1	0.55 ± 0.1*	0.62 ± 0.1†	0.06 ± 0.1
Total cholesterol (mg/dl)	163.7 ± 36.2	152.9 ± 37.2†	−10.7 ± 15.9	159.0 ± 28.5	145.5 ± 25.1†	−13.5 ± 16.5
HDL cholesterol (mg/dl)	43.3 ± 7.9	42.9 ± 9.7	−0.4 ± 4.5	43.3 ± 10.5	46.0 ± 11.8†	2.7 ± 4.5***
LDL cholesterol (mg/dl)	91.2 ± 29.0	88.4 ± 32.4	−2.8 ± 19.7	93.8 ± 22.0	80.2 ± 16.5†	−13.5 ± 15.9
VLDL cholesterol (mg/dl)	25.8 ± 13.9	21.6 ± 11.7	−4.2 ± 9.0	21.9 ± 10.7	19.2 ± 11.6	−2.7 ± 6.5
Triglyceride (mg/dl)	151.0 ± 117.3	108.2 ± 58.2†	−42.8 ± 77.1	109.8 ± 53.6	96.3 ± 57.7	−13.5 ± 32.7
AST (U/l)	25.8 ± 7.0	24.0 ± 8.3	−1.8 ± 6.5	21.8 ± 4.5*	22.0 ± 6.4	0.2 ± 5.8
ALT (U/l)	37.2 ± 22.1	27.5 ± 15.0†	−9.7 ± 13.8	21.5 ± 11.4*	21.8 ± 12.9	0.3 ± 8.0***
GGT (U/l)	28.9 ± 18.1	22.7 ± 13.5†	−6.2 ± 6.4	22.1 ± 15.4*	19.0 ± 10.5	−3.1 ± 9.2
Adiponectin (μg/l)	7.2 ± 3.8	9.6 ± 4.2†	2.4 ± 3.3	7.6 ± 3.5	8.9 ± 5.7	1.3 ± 3.8
Leptin (ng/ml)	46.4 ± 31.1	27.9 ± 28.0†	−18.4 ± 23.2	39.5 ± 26.0	36.1 ± 27.7	−3.4 ± 28.0
Lep/Adipo ratio	8.72 ± 10.3	4.47 ± 6.9†	−24.0 ± 84.2	6.93 ± 6.5	6.82 ± 8.5	−0.11 ± 6.4

Reference values: glucose (60–110 mg/dl), insulin (<20 μU/ml), HOMA-IR (<2.0), QUICKI (>0.339), total cholesterol (<170 mg/dl), TG (33–129 mg/dl), HDL cholesterol (>38 mg/dl), LDL cholesterol (<130 mg/dl), VLDL cholesterol (10–50 mg/dl), AST (10–40 U/l), ALT (10–35 U/l), and GGT (17–30 U/l) as previously described by Schwimmer et al. [25]. Leptin values between 1 and 20 ng/ml for males and between 4.9 and 24 ng/ml for females described by Gutin et al. [24]

NAFLD non-alcoholic fatty liver disease, *HOMA-IR* homeostasis model assessment insulin-index resistance, *HLD* high-density lipoprotein, *LDL* low-density lipoprotein, *VLDL* very-low-density lipoprotein, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *GGT* γ-glutamyl transferase, *QUICKI* quantitative insulin sensitivity check index

* Statistical differences in baseline values between NAFLD and non-NAFLD group ($P < 0.05$)

** Statistical differences in year values between NAFLD and non-NAFLD group ($P < 0.05$)

*** Statistical differences in Δ values between NAFLD and non-NAFLD group ($P < 0.05$)

† Statistical differences in basal vs year (therapy effect) in the same group ($P < 0.05$)

inflammatory state, which can lead to increase osteogenesis and bone metabolism by mechanical, structural, and cellular processes involving trabecular bone formation and decrease bone reabsorption [7, 39–41]. However, a study with a longer time approach is suggested, once we were not able to show an improvement of BMD.

For the adolescents with reduced HOMA-IR and increased insulin sensitivity values after therapy, is possible that the improved BMD was a direct result of improved insulin resistance, as it has been previously present that obese adolescent with NAFLD decreased BMD that is, inversely correlated with insulin resistance [14]. It was showed in this study from the analysis of both groups, that BMC is inversely correlated with insulin resistance and this emphasizes the importance of the clinical approach for the maintenance of bone health in this population. Moreover, additional studies have reported that NAFLD is significantly associated with reduced biological effects of insulin and have shown decreased insulin sensitivity in obese children and a significant difference in insulin sensitivity between children with and without NAFLD [42, 43].

It is plausible that insulin resistance may at least partially explain the association between obesity and suboptimal bone mass [1]. Insulin plays a primary role in bone formation by, acting as an anabolic agent in bone and preserving and increasing BMD and bone strength [44]. In a cohort of overweight Hispanic-American children an inverse association between BMC and insulin resistance was demonstrated [45]. Similarly, in a study of overweight pre-pubertal children, children with pre-diabetes were found to have a reduced bone mass when compared to children with glucose levels within the reference range [46].

These studies indicate that abnormal glucose regulation has a negative effect on the growing skeleton. The mechanism for the potential negative effect of insulin resistance on bone development is currently unknown although hypotheses include an increased calcium excretion [47], increased concentrations of advanced glycation end-products in collagen [48], a disruption in the growth hormone-insulin-like growth factor axis, and increased inflammation [49].

Table 3 Correlations analyses

Groups	Δ Variables	*r*	*P*
Total ($n = 40$)	BMD (g/cm^2)		
	Total fat (%)	0.30	0.01
	Visceral fat (cm)	−0.60	0.66
	Subcutaneous fat (cm)	0.30	0.05
	Insulin (μU/ml)	−0.60	0.30
	BMC (g)		
	HOMA-IR	−0.34	0.02
	Total body mass (kg)	0.34	0.16
	Visceral fat (cm)	−0.55	0.84
	VLDL cholesterol (mg/dl)	0.34	0.15
	HOMA-IR		
	Leptin (ng/ml)	−0.34	0.02
NAFLD ($n = 18$)	BMD (g/cm^2)		
	Total fat (%)	0.56	0.01
	Total cholesterol (mg/dl)	0.48	0.03
	HOMA-IR		
	Lep/Adipo ratio	0.72	0.001
Non-NAFLD ($n = 22$)	BMD (g/cm^2)		
	Total fat (kg)	0.50	0.01
	BMC (g)		
	HOMA-IR	−0.43	0.04

Statistical significance $P < 0.05$, *NAFLD* non-alcoholic fatty liver disease, *BMD* bone mass density, *BMC* bone mass content, *HOMA-IR* homeostasis model assessment insulin-index resistance, *VLDL* very-low-density lipoprotein, *Lep/Adipo ratio* leptin/adiponectin ratio

One important aspect, which has been demonstrated from animal studies, is the existence of a "bone-fat-pancreas" axis that regulates energy homeostasis, coordinates energy partitioning between bone and adipose tissue and impacts insulin sensitivity. When recombinant osteocalcin was administered to the animals, improvements in glucose tolerance and insulin secretion were observed [44]. The novel relationships aforementioned between osteocalcin and glucose-insulin metabolism appear to be regulated by leptin [50].

In our study, a negative correlation was found between HOMA-IR and the variation in leptin concentration. Leptin is an adipocyte-derived hormone that is strongly and positively correlated with fat mass levels [51]. In addition, recently states of hyperleptinemia were associated to inflammatory process, in both animals and humans. In animals low doses of leptin can stimulate bone formation and prevent bone loss, but higher concentrations of leptin actually suppress bone formation and increase bone resorption [52]. In our study, it was demonstrated a reduction of leptin concentration in the NAFLD group and this reduction could be responsible of the improvement of BMC, but not in BMD, probably due 1 year of interdisciplinary therapy was not enough and BMD could need more time to increase. The relationship between leptin and bone mass is complex, leptin exerts opposite effects on the skeleton. Thus, a hyperleptinemia state might influence bone formation and bone resorption [53]. A correlation study failed to detect an independent relationship between serum leptin concentrations and total body or regional BMC and BMD [54].

Therefore, a possible and plausible explanation by which greater levels of adiposity may inhibit the accumulation of bone mass during growth is via hyperleptinemia, commonly observed in obese population. In fact, in the present investigation hyperleptinemia was observed in both analyzed groups at baseline. Interestingly, after 1 year of interdisciplinary therapy, there was a reduction in leptin levels and HOMA-IR, which probably contributed to attenuate the negative effect of hyperleptinemia and insulin resistance on bone metabolism. Corroborating with this hypothesis, only NAFLD group present in the regression analyses that Δ HOMA-IR was an independent predictor

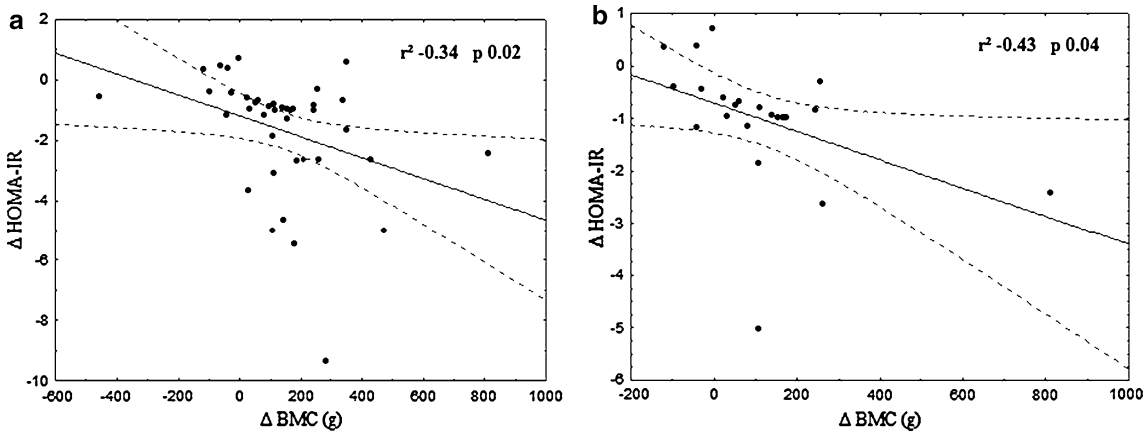

Fig. 2 **a, b** Relationship between Δ BMC and Δ HOMA-IR in total obese adolescents (**a**) and in *non-NAFLD* obese adolescents (**b**)

for changes in Δ BMC (Fig. 2a, b). Thus, the fact that the adipokines could be implicated in the bone control could be suggested only in this group.

Another notable result from this study was the increase in adiponectin concentration only in the NAFLD group favoring its anti-inflammatory effects after therapy. The interaction of this adipokine with bone metabolism remains controversial [55–58]. Although it has been demonstrated that adiponectin can reduce osteoclasts numbers and can favor bone formation via activation of osteoblastogenesis [59]. In agreement, multiple clinical, meta-analysis and genetic studies have correlated hipoadiponectinemia with insulin resistance and higher adiponectin concentrations associated with lower risk of type II diabetes [56–59]. These findings support a primary role for adiponectin in preventing metabolic disease in humans [60].

Finally, we were able to show a significant reduction in the body mass and visceral fat mass and a reduction in the prevalence of the NAFLD steatosis (100 to 33%). Visceral fat is an independent risk factor for NAFLD prevalence in obese adolescents, suggesting the important role of adipose tissue metabolism. Moreover, the expansion of visceral fat can promote an increase in the pro-inflammatory adipokines secretion and reduction in the adiponectin, a potent anti-inflammatory adipokine known to be also a cardiovascular risk factor [61].

Together, the data of the present investigation highlight the importance of the weight loss in the balance between pro- and anti-inflammatory adipokines and the regulation of bone metabolism. A weight loss could reduce leptin levels and leptin/adiponectin ratio improving BMC in NAFLD obese adolescents.

In conclusion, 1 year of interdisciplinary weight loss therapy for obese adolescents with or without NAFLD, could regulate bone mineral metabolism as result of an increased BMC. These data are important in clinical practice to improve the control of related multifactor risks, linking obesity with NAFLD in adolescents.

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