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Effects of Conjugated Linoleic Acid and Metformin on Insulin Sensitivity in Obese Children: Randomized Clinical Trial

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Context: Insulin resistance precedes metabolic syndrome abnormalities and may promote cardiovascular disease and type 2 diabetes in children with obesity. Results of lifestyle modification programs have been discouraging, and the use of adjuvant strategies has been necessary.

Objective: This study aimed to evaluate the effects of metformin and conjugated linoleic acid (CLA) on insulin sensitivity, measured via euglycemic-hyperinsulinemic clamp technique and insulin pathway expression molecules in muscle biopsies of children with obesity.

Design: A randomized, double-blinded, placebo-controlled clinical trial was conducted.

Setting: Children with obesity were randomly assigned to receive metformin, CLA, or placebo.

Results: Intervention had a positive effect in all groups. For insulin sensitivity Rd value (mg/kg/min), there was a statistically significant difference between the CLA vs placebo (6.53 ± 2.54 vs 5.05 ± 1.46, $P = 0.035$). Insulinemia and homeostatic model assessment of insulin resistance significantly improved in the CLA group ($P = 0.045$). After analysis of covariance was performed and the influence of body mass index, age, Tanner stage, prescribed diet, and fitness achievement was controlled, a clinically relevant effect size on insulin sensitivity remained evident in the CLA group (37%) and exceeded lifestyle program benefits. Moreover, upregulated expression of the insulin receptor substrate 2 was evident in muscle biopsies of the CLA group.

Conclusions: Improvement of insulin sensitivity, measured via euglycemic-hyperinsulinemic clamp and IRS2 upregulation, favored patients treated with CLA. (*J Clin Endocrinol Metab* 102: 132–140, 2017)

Obesity is a multifactorial disease with high prevalence in Mexico. According to the 2012 National Survey of Health and Nutrition, the prevalence of overweight and obese children and adolescents in Mexico is 34% (1). Insulin resistance has been recognized as the main physiopathological event preceding

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metabolic syndrome abnormalities and may promote cardiovascular disease and type 2 diabetes in individuals with obesity (2). Lifestyle modification through healthy food selection and consumption, a regular physical activity program, and optimal sleep hygiene have been proposed as the gold standard of care in these individuals. Unfortunately, the compliance and success of these strategies are usually disappointing (3, 4), making pharmacological approaches somewhat necessary. Metformin (MET) is a biguanide used for the treatment of type 2 diabetes in children and adolescents due to its ability to decrease hepatic glucose production and increase peripheral insulin sensitivity. MET has been proposed as an adjuvant treatment in pediatric obesity efforts, especially in the presence of insulin resistance and its comorbidities. MET has beneficial effects on weight reduction and insulin resistance in obese nondiabetic individuals (5, 6).

Conjugated linoleic acid (CLA) is a group of isomers of linoleic acid, which are synthesized in the cud of ruminant animals by fermentative bacteria (7). CLA is present in dairy products, meat, and fat from beef and lamb. The most common CLA isomer contained in these products is *cis*-9,*trans*-11, which can be commercially synthesized from linoleic acid–rich oils and prepared as a 50% mixture with the *trans*-10,*cis*-12 isomer (8). Several studies have acknowledged the beneficial effects of CLA isomers on body composition (9, 10), immune response (11), bacterial-induced colonic inflammation (12, 13), as well as improvements in insulin sensitivity and lipid metabolism in experimental animals and humans (9). Additionally, CLA purportedly reduces fatty acid synthesis in adipocytes, suggesting that this supplement decreases fat deposition, directly contributing to an improvement in body composition in adults and children (14). Nonetheless, the impact of CLA on human health and disease is still controversial and research on this matter continues.

Based on the current obesity frequency in Mexico, and considering the limited and discouraging outcomes of intervention programs, adjuvant strategies must be installed. The objective of the present study was to evaluate the effects of MET and CLA on insulin sensitivity, measured via the euglycemic-hyperinsulinemic clamp technique (EHCT), in children with obesity.

Subjects and Methods

We performed a randomized, double-blinded, 16-week placebo (PLB)-controlled trial in the Pediatric Obesity Clinic at the Pediatrics Department of Hospital General de México (Mexico City, Mexico).

Patients with obesity aged 8 to 18 years who had not been previously intervened and had optimal psychological health were included in the study. Obesity was defined using Centers for Disease Control and Prevention criteria [body mass index (BMI) $\geq$ 95th percentile]. Exclusion criteria included

BMI $\geq$ 35 kg/m^2, genetic or endocrine obesity, a systemic illness, diabetes or prediabetes (according to American Diabetes Association criteria) (15), and the use of weight loss medications that could modify lipids and glucose concentrations. The study (no. DI/11/311/04/108) was approved by the hospital's institutional review board; additionally, it was registered in ClinicalTrials.gov (no. NCT02063802).

All participants were included in the standardized healthy lifestyle program addressed to children and their parents. This 4-month program consisted of a monthly visit that included a 1-hour structured physical activity session (coordinated by a physical trainer), followed by a psychoeducational group session. The following information was presented to all participants: (a) description of a balanced and healthy nutrition, (b) emotion-related eating behavior and family support, (c) the benefits of physical activity, and (d) obesity-related comorbidities. These sessions were coordinated by nutritionists, psychologists, pediatricians, pediatric endocrinologists, and a physical trainer. Afterward, all patients held a medical consultation to evaluate their anthropometry and medical condition, as well as their progression and acquisition of skills and their compliance to the program. At the beginning of the intervention a complete nutritional evaluation was performed and a diet based on age, pubertal stage, and physical activity requirements, according to the World Health Organization and Food and Agricultural Organization guidelines, was prescribed (16). The recommended diet composition was 55% carbohydrates, 20% proteins, 25% lipids (<7% saturated fat, <300 mg/d cholesterol, and <1% *trans* fat), and <3 g of salt per day. Participants filled out a 24-hour nutritional recall questionnaire during the 3 days prior to their follow-up appointment to assess diet compliance. All patients were encouraged to participate in sports activities at last 5 days a week and for a minimum of 60 minutes.

To evaluate physical activity compliance, we tested fitness achievement using the Harvard step test modified for the pediatric population and a physical fitness score was calculated (17); evaluations were applied at baseline and at the postintervention state. The overall intervention compliance was evaluated through anthropometric, metabolic, and fitness parameter modifications, as well as through the acquisition of healthy behavior knowledge.

Clinical trial design

This trial was conducted in accordance with the Declaration of Helsinki and adhered to Good Clinical Practice Guidelines issued by the International Conference of Harmonization. The children and their parents provided written informed assent/consent. Eligible patients were included in the lifestyle intervention program (LIP) and randomized to receive either MET (1 g/d), CLA containing 50:50 isomers *c9,t11* and *t10,c12* (3 g/d), or PLB (1 g/d) 3 times a day for 16 weeks. Visits were scheduled monthly. Diet, exercise, and medication compliance, as well as anthropometric variables, were recorded during each visit. The final evaluation was similar to baseline; EHCT and skeletal muscle biopsies were performed at the postintervention state. Patients were eliminated when they showed poor compliance to medication (<80% or >100%) or intolerance, or when $\geq$1 workshop sessions were missed.

Anthropometric and metabolic evaluation

Baseline evaluation consisted of complete anthropometric and body composition analysis. Height and weight were obtained

with participants in light clothes and without shoes, using a standardized stadiometer and mechanical scale. A 12-hour fasting blood sample was drawn. Laboratory measurements included glucose, lipid profile, and aminotransferases that were analyzed enzymatically with the use of commercially available reagents. Insulin was measured using Bio-Plex Pro human diabetes insulin immunoassay by Bio-Rad (Hercules, CA). Fasting insulin resistance and sensitivity surrogated indexes were calculated as follows: homeostatic model assessment of insulin resistance (HOMA-IR) = [fasting plasma insulin (μU/mL) $\times$ fasting plasma glucose (mmol/L)]/22.5, and quantitative insulin sensitivity check index (QUICKI) = 1/[log fasting plasma insulin (μU/mL) + log fasting plasma glucose (mg/dL)].

Clamp procedure

A 2-hour euglycemic-hyperinsulinemic clamp was performed (18) and executed during a 12-hour fasting condition. Intravenous catheters were inserted in the right and left forearm vein, one in a retrograde direction, and warmed in a box that was designed for this purpose (Kepis Keipis One Device, unpublished data). This device allowed the introduction of the complete forearm and maintenance of adequate high temperature and humidity that provided an arteriovenous shunt for blood sample supply while avoiding burns. The additional vein was used to infuse insulin and 20% dextrose solution at variable rates. Intravenous crystalline insulin (Humulin; Eli Lilly & Co., Indianapolis, IN) was used. A priming insulin dose of 120 mIU/m^2 of body surface (bs) per minute at time 0 was administered after 1 hour of baseline and during the first 5 minutes. Thereafter, the infusion was gradually reduced to 60 mIU/m^{2}bs/min up to minute 10 and maintained through the end of the clamp. Glucose infusion started at minute 5 (5 mg/kg/min in all the patients according to information obtained during the standardization procedure). The samples were obtained every 5 minutes, and glucose infusion was dynamically modified to clamp plasma glucose at 85 to 95 mg/dL.

The rate of glucose disposal (Rd) was calculated and adjusted during the last 30 minutes of the clamp when plasma glucose stabilized at a fixed range.

Primary endpoints included the postintervention insulin resistance state defined as the Rd value (mg/kg/min) measured via EHCT, as well as the evaluation of surrogate indexes of insulin resistance and sensitivity (insulinemia, HOMA-IR, and QUICKI). The expression of insulin receptor substrates IRS1, IRS2, and IRS4 in muscle biopsies complemented the insulin resistance study. Secondary objectives were modifications of anthropometric and metabolic parameters. Moreover, medication safety and tolerability were important outcomes.

Muscle biopsies

The participation of MET and CLA on the insulin signaling pathway was explored with muscles biopsies from the vastus lateralis performed under local anesthesia after 16 weeks of intervention. An incision with a no. 11 surgical blade was made to insert an 8 swg (4.0 mm) Bergstrom needle (Ultramed, Milton, ON, CA).

RNA isolation

Total RNA was isolated from biopsies samples using an RNeasy fibrous tissue minikit for muscle and an RNeasy lipid tissue minikit for adipose tissue (Qiagen, Valencia, CA) following the manufacturer's protocol. RNA concentration was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). Integrity was evaluated by agarose gel electrophoresis using a vertical chamber Enduro (Labnet International, Edison, NJ) and the UltraSlim LED Illuminator SLB-01 (Maestrogen, Las Vegas, NV).

Genetic expression of insulin receptors

The genetic expression patterns of IRS1, IRS2, and IRS4 were studied in 14 and 17 muscular tissue biopsies obtained from the MET and CLA groups, respectively. Quantitative reverse transcription polymerase chain reaction array (human insulin signaling pathway, RT^2 Profiler, PAHS-030Z, Qiagen) was performed. Complementary DNA was prepared using an RT^2 polymerase chain reaction array first-strand kit (Qiagen) according to the manufacturer's instructions. Normalization was computed with ACTB, B2M, GAPDH, HPRT1, and RPLP0. The expression patterns observed in the MET and CLA groups were compared with muscular tissue samples from the PLB group (n = 17) used as calibrator. The differential gene expression was calculated using the Qiagen software polymerase chain reaction analyzer through the $2^{-\Delta\Delta Ct}$ analysis, and a 2.5-fold change cut-off ($P < 0.05$) was considered.

Statistical analysis

Descriptive statistics for all numerical variables are reported as the mean and standard deviation and standard error of the mean (SEM) for contrasts as indicated in the text or figures. Contrast among treatment groups was assessed by analysis of variance and analysis of covariance (ANCOVA) for adjustment by confounding variables. Post hoc analysis and the multiple contrast hypothesis corrected by Fisher's least significant distance were executed. The η^2 effect sizes obtained from ANCOVAs were transformed to Cohen's d. χ^2 Analyses were also executed to evaluate differences in proportion among groups. SPSS software version 22 (IBM, Armonk, NY) was used to conduct the statistical analyses. A probability of α error of <5% was considered as statistically significant.

Results

Participants and demographics

Enrollment occurred from August 2012 to July 2014. One hundred ninety-eight individuals were potentially eligible; 83 met inclusion criteria, signed consent and assent forms, and were randomized to receive MET (n = 24), PLB (n = 30), and CLA (n = 29). Fifty patients completed the 16-week intervention; 1 external outlier was identified and excluded during the analysis (PLB group). In 1 case, EHCT performance was technically impossible (CLA group). For this reason, we report the results of 48 executed clamps (Fig. 1). Throughout the study, 1 patient was eliminated when a preexisting lipoma was surgically removed without notifying the research team (PLB group); a second patient with psychosocial anomalies and suspected pregnancy was eliminated as well (PLB group). Twenty-nine patients were eliminated due to poor medication compliance or due to lack of interest (MET, n = 10; PLB, n = 10; and CLA, n = 9). Pubertal development (Tanner stage 1,

defined as prepubertal; Tanner stages 2 to 3, defined as early puberty; and Tanner stages 4 to 5, defined as late puberty) was assessed after a clinical inspection of the mammary glands, testes volume, and pubic hair. Demographic and baseline characteristics were similar among the groups (Table 1).

Anthropometric and metabolic results

No significant differences were observed in baseline anthropometric and metabolic parameters or insulin resistance measured by surrogate indexes of insulin resistance (fasting insulinemia, HOMA-IR, and QUICKI). Distribution of Tanner stage status did not differ among the groups (χ^2 test, $P = 0.415$).

The overall impact of the intervention showed a positive effect on weight, height, BMI, and waist circumference, as well as on surrogated indexes of insulin resistance and physical fitness score in all of the groups (Table 2). No statistically significant differences were observed in these parameters between treatment groups. No differences were evident when comparing surrogate indices of insulin resistance during the postintervention phase among the groups.

Insulin sensitivity measured by EHCT

The primary outcome, insulin sensitivity, calculated as the Rd value, showed significant difference between the CLA group compared with PLB (6.53 ± 2.54 vs 5.05 ± 1.46, $P = 0.035$, Cohen's d effect size of 74%) (Table 3). Moreover, fasting insulinemia (Fig. 2) and HOMA-IR (Fig. 3) significantly decreased in the CLA group ($P = 0.04$). The adjusted analysis for controlling the influence of modifying or confounding variables such as BMI, change in BMI, age, Tanner stage, as well as dietary and physical program compliance, over the Rd value, showed that the Tanner stage had an independent effect over the Rd value ($P < 0.001$). When ANCOVA was executed and the aforementioned variables were controlled, no statistically significant differences were found between the three groups with regards to Rd value. Nonetheless, a clinically relevant effect size remained evident when comparing the CLA and PLB groups (Cohen's d effect size of 37%), suggesting a decrease in insulin resistance in patients receiving CLA. The effect size of MET vs PLB was 10% (5.72 ± 3.1 vs 5.38 ± 3) and that of MET vs CLA was 20% (5.72 ± 3.1 vs 6.34 ± 2.8), favoring the
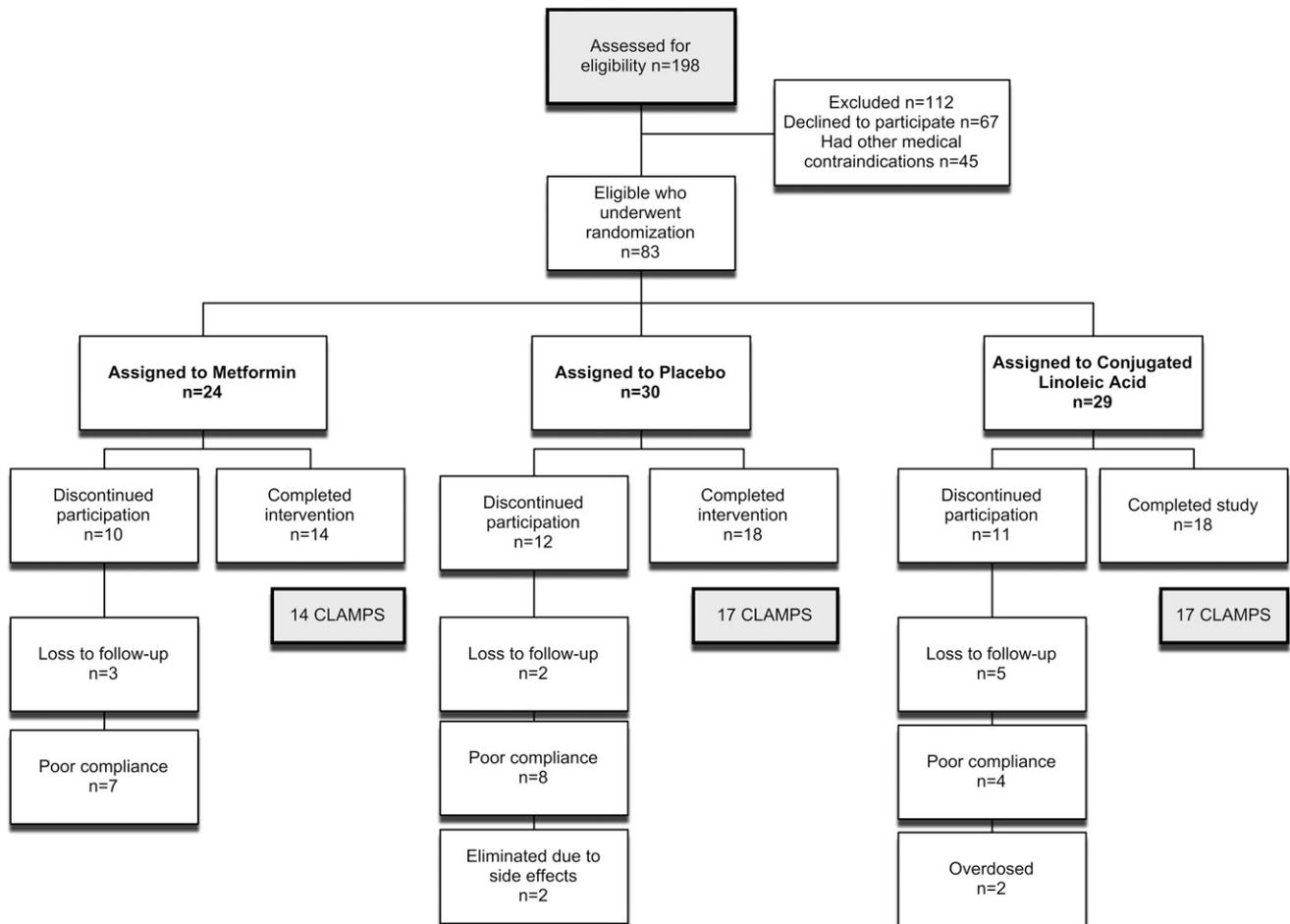
Figure 1. Flowchart representing the number of subjects at study enrollment and study termination. Fourteen clamp studies were conducted in the PLB group, 17 in the MET-treated group, and 17 in the CLA-treated group.

Table 1. Participant Characteristics at Randomization

	MET (n = 14)	Placebo (n = 17)	CLA (n = 17)	P
Age, y	11.43 (2.1)	12.59 (2.62)	11.41 (2.71)	0.31
Weight, kg	63.25 (12.6)	70.15 (13.11)	62.11 (18.47)	0.26
Height, cm	148.4 (10)	153.4 (12)	148.6 (13)	0.42
BMI, kg/m^2	28.54 (2.8)	28.79 (2.8)	27.48 (3.7)	0.45
Waist circumference, cm	87.12 (7.6)	89.43 (8.45)	87.17 (11.2)	0.71
Glucose, mg/dL	87 (7)	87 (8.2)	85 (8.1)	0.62
Total cholesterol, mg/dL	167 (27)	167 (28)	162 (23)	0.79
HDL cholesterol, mg/dL	41 (10)	37 (8)	38 (8)	0.42
LDL cholesterol, mg/dL	107 (24)	106 (24)	100 (25)	0.67
Triglycerides, mg/dL	150 (62)	161 (60)	159 (84)	0.89
Physical fitness score (Harvard test)	81 (56)	83 (45)	85 (66)	0.98
Insulin, μU/mL	43.5 (22)	37.1 (17)	42.5 (27)	0.25
HOMA-IR	9.42 (4)	8.07 (4)	9.2 (6)	0.68
QUICKI-IS	0.63 (0.08)	0.64 (0.06)	0.66 (0.15)	0.54

Data are expressed as mean (standard deviation).

Abbreviation: LDL, low-density lipoprotein.

CLA-treated group. However, these effect sizes were not clinically relevant.

We analyzed the changes between initial and final serum triglycerides and high-density lipoprotein (HDL) cholesterol by ANCOVA. For these particular variables, no Tanner or change in BMI modified postintervention levels. Furthermore, HDL cholesterol and triglyceride baseline levels did show an influence over the final levels.

Lipid profile and adverse effects

Patients in the CLA group had a statistically significant increase in serum triglycerides when compared with MET (169.8 ± 69 vs 113.1 ± 27, $P = 0.027$), but not significant when compared with PLB ($P = 0.13$). Moreover, HDL levels were lower in the CLA group when compared with MET (36.8 ± 5.4 vs 44.86 ± 8.7, $P = 0.009$), whereas there were no differences when compared with PLB ($P = 0.26$). The main differences favoring MET treatment

over the lipid profile were evident only when compared with CLA.

Nonserious adverse events most commonly reported were abdominal pain, diarrhea, dizziness, headache, nausea, and gastritis. The frequency and severity of symptoms were similar in the three groups (analysis of variance, $P = 0.314$; χ^2 test, $P = 0.28$). Patients exhibiting lack of compliance and/or dropout did not show a difference between groups. Additionally, a Little's missing completely at random analysis ($P > 0.13$) was conducted to ensure that patient elimination was actually random and homogeneous in all of the groups.

Muscle biopsies' analyses

The analyses of IRS1, IRS2, and IRS4 revealed that only IRS2 was modulated in the CLA group, showing a 3.56-fold increase compared with the control group ($P = 0.043$). The rest of the genes did not show statistically

Table 2. Overall Impact of Intervention Regarding Anthropometric, Metabolic, and Insulin Resistance Outcomes in All Patients

	Baseline (n = 48)	4 Months Postintervention (n = 48)	P
Weight, kg	65.29 (2.2)	62.51 (2.15)	<0.001
Height, cm	150.2 (1.75)	151.7 (1.66)	<0.001
BMI, kg/m^2	28.25 (0.45)	26.56 (0.51)	<0.001
Waist circumference, cm	87.9 (1.32)	83.8 (1.24)	<0.001
Glucose, mg/dL	87 (1.13)	85 (0.85)	0.28
Total cholesterol, mg/dL	167 (3.75)	161 (4.21)	0.07
HDL cholesterol, mg/dL	39.2 (1.27)	40.4 (1.21)	0.24
LDL cholesterol, mg/dL	105 (3.53)	100 (3.57)	0.056
Triglycerides, mg/dL	154 (9.96)	139 (9.73)	0.22
Insulin, μU/mL	40.9 (3.26)	34.5 (2.95)	0.025
HOMA-IR	8.86 (0.77)	7.27 (0.63)	0.018
QUICKI	0.64 (0.01)	0.68 (0.01)	0.014
Physical fitness score (Harvard test)	83.82 (7.96)	124.0 (5.44)	<0.001

The P values were determined by a paired-samples Student t test. Data are expressed as mean (SEM).

Abbreviation: LDL, low-density lipoprotein.

Table 3. Characteristics of Main Interest Variables After 4 Months of Intervention

	MET (n = 14)	Placebo (n = 17)	CLA (n = 17)	P
Weight, kg	60.85 (3.41)	66.48 (3.19)	59.91 (4.34)	0.39
Height, cm	149.89 (2.45)	154.82 (2.82)	150.27 (3.19)	0.40
BMI, kg/m^2	26.17 (0.99)	27.53 (0.78)	25.92 (0.90)	0.37
Waist circumference, cm	82.31 (1.87)	84.96 (1.77)	83.92 (2.68)	0.70
Prescribed diet compliance, %	92.04 (6.15)	83.78 (6.21)	83.09 (3.56)	0.43
Physical fitness score (Harvard test)	103.82 (16)	132.9 (13.09)	132.79 (14.61)	0.29
Rd value, mg/kg/min	5.57 (0.47)[a,b]	5.05 (0.35)[b]	6.53 (0.61)[a]	0.035
Insulin, μU/mL	40.5 (6.37)	32.3 (4.11)	31.6 (5.05)	0.42
HOMA-IR	8.53 (1.36)	6.83 (0.88)	6.68 (1.08)	0.45
QUICKI-IS	0.65 (0.02)	0.69 (0.02)	0.71 (0.02)	0.38
HDL cholesterol	44.86 (2.33)[a]	40.00 (2.15)[a,b]	36.87± (1.35)[b]	0.031
Triglycerides, mg/dL	113.14 (7.26)[b]	134.12 (20.36)[a,b]	169.80 (15.93)[a]	0.027

Data are expressed as mean (SEM).

[a,b]Homogeneous groups by Fisher's least significant difference contrast.

significant differences. These data support the fact that CLA has a critical effect in the molecular insulin pathway through the upregulation of ISR2. This mechanism might be related to optimal glucose uptake observed in CLA-treated patients.

Discussion

This study supports that CLA improves insulin sensitivity, as measured by EHCT in a group of obese children, and exceeds LIP benefits.

Because the prevalence of metabolic syndrome in our pediatric clinic averages 35% and confers an 11-fold risk of diabetes during early adult life (19), the exploration of conventional and pharmacological strategies focusing on improving the insulin sensitivity level is imperative. Recent studies have revealed that MET has important effects on insulin sensitivity when compared with PLB, and its use in nondiabetic, obese individuals has been massively extended (6, 20, 21). A systematic review conducted by Brufani et al. (6) revealed a significant but moderate benefit of MET on weight reduction and fasting insulin sensitivity compared with PLB or lifestyle interventions alone. Nonetheless, when these outcomes were evaluated by frequently sampled intravenous glucose tolerance test (22) or hyperglycemic clamp technique (23), no significant differences were reported. Wiegand et al. (5) demonstrated in a randomized PLB-controlled trial a beneficial effect of MET on the insulin sensitivity index in obese, insulin-resistant adolescents; however, no differences in body composition, weight, or BMI were found. We found in our study a significant improvement in all anthropometric parameters, including weight, BMI, waist circumference, and body composition (fat mass and fat-free mass, data not shown); nonetheless, these results were not significantly different among the treatment groups. To our knowledge, no randomized PLB-controlled trial using

EHCT had been executed for the evaluation of MET benefits on insulin sensitivity in children. In the present study, we found no differences on Rd value (mg/kg/min) when comparing MET and PLB in the postintervention period. These data could be consistent with the final results of the Diabetes Prevention Program Research Group (24) that showed that diabetes incidence was better reduced in a LIP group compared with PLB. Nonetheless, improvement in terms of BMI, waist circumference, HDL cholesterol, and triglycerides, with considerable effect sizes (72%, 65%, 37%, and 55%, respectively), favored patients treated with MET in our study.

Several studies have proposed beneficial effects of CLA isomers on body composition, inflammation, and insulin sensitivity, promoting differentiation, lipid metabolism regulation, and apoptotic mechanisms in adipocytes (10–12). Interestingly, evidence has suggested that the trans-10,cis-12 isomer of CLA might induce insulin resistance, whereas the CLA mixture has beneficial effects on body composition and insulin sensitivity. Risérus et al. (25) demonstrated that trans-10,cis-12 isomer–treated subjects presented insulin and glucose increases and decreased HDL and insulin sensitivity measured by 2-hour EHCT compared with PLB or CLA mixture-treated groups. No differences were observed when comparing PLB and CLA mixture-treated individuals. In our study, we showed that the CLA mixture was associated with a clinically relevant effect size (37%) over the Rd value of insulin sensitivity. The confounding variables included in the ANCOVA model showed a decline on group differences. Among these adjustments, Tanner stage was the main variable that modified insulin sensitivity, and despite our small sample size, the effect size of CLA on the Rd value remained.

Despite the fact that several CLA isomers might have deleterious effects on insulin sensitivity and resistance, certain mixtures may neutralize negative effects and even

Pre and Post treatment Insulin levels
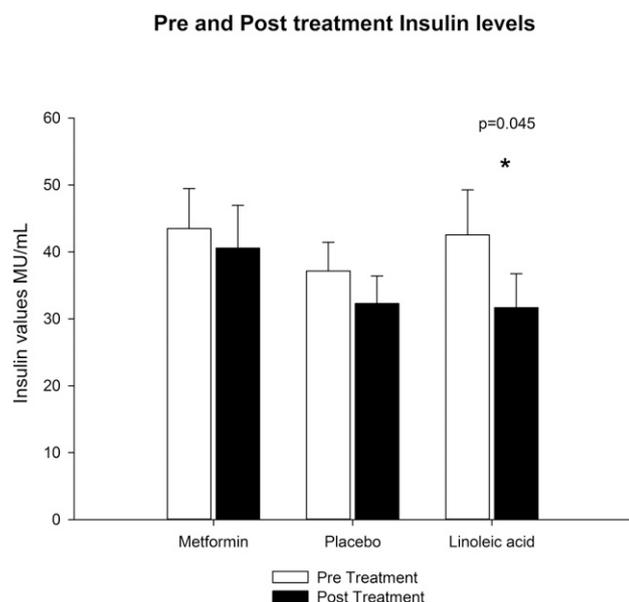
Figure 2. Fasting insulin serum concentrations were significantly lower in the CLA-treated group at study termination, whereas no differences were found in the MET-treated patients or in the PLB group. The error bars show SEM.

induce a synergistic positive response on these parameters, as well as on metabolic and anthropometric values. Some effects of the *trans*-10,*cis*-12 isomer promote a blunted glucose uptake that depends on decreased expression of glucose transporter 4 (GLUT-4) (26). Moreover, decreased incorporation of free fatty acids into the cells may be induced by CLA, a mechanism that could be related to diminished expression of peroxisome proliferator-activated receptor-γ in adipocytes (27). CLA has been proposed as an apoptotic accelerator of adipocytes in mammals that liberates and increases fatty acid oxidation elsewhere in the body (28). Evidence of deleterious effects has mainly been reported in animal models, in which administered doses of CLA are superior to those used in humans (0.2 to 3 g/kg vs 0.015 to 0.1 g/kg, respectively) (29). These effects, if present in humans, could be hyperglycemia and hyperlipidemia, which may predispose an individual to diabetes and nonalcoholic fatty liver disease (30, 31). However, few studies have been published regarding the molecular mechanisms of CLA in skeletal muscle that could explain increased glucose uptake in our treated patients. On this matter, Vaughan *et al.* (32), using a rabdomyosarcoma cell line, have reported that omega-3 fatty acids and CLA activate mitochondrial proliferation and glycolytic activation pathways probably by apoptosis induction and subsequent upregulation of GLUT-4. Furthermore, animal models have shown the beneficial effects of CLA on insulin sensitivity and overexpression of peroxisome proliferator-activated receptor-γ and GLUT-4 in the muscle of supplemented rats (27). In the present study we

were able to demonstrate that postintervention IRS2 expression in the skeletal muscle was significantly upregulated in CLA-treated patients. To our knowledge, no studies have been published regarding the effects of CLA or CLA–isomer mixtures on insulin receptor substrate molecules. Xu *et al.* (33) reported that MET upregulates insulin receptor β expression and the downstream IRS2/phosphatidylinositol 3-kinase/Akt signaling transduction in an insulin-resistant rat model of nonalcoholic steatohepatitis and cirrhosis. Our results evidenced a nonsignificant but marginal ($P = 0.055$) IRS2 upregulation in MET-treated children. The insulin-sensitizing effects of MET have been mainly described in liver tissue. Although CLA effects have been mainly focused on adipose tissue modeling, the present study demonstrates that molecular mechanisms, particularly IRS2 upregulation, might mediate insulin-sensitizing effects on skeletal muscle. This phenomenon could explain the increased glucose infusion rate tolerability in our patients treated with CLA throughout the EHCT. Moreover, significant HOMA-IR improvement observed only in CLA-treated patients denotes a significant performance in skeletal muscle that promotes a lower pancreatic insulin secretion.

A recently published meta-analysis demonstrated that the deleterious effects of CLA consumption might be negligible, whereas its benefits, although subtle, seem to be clinically relevant regarding weight and fat mass loss (34). In our study, BMI improvement was significant in all groups, although not significantly different among them. Nonetheless, MET displayed the highest effects over BMI (72%, compared with 43% in the PLB group and 41% in

Pre and Post treatment HOMA-IR
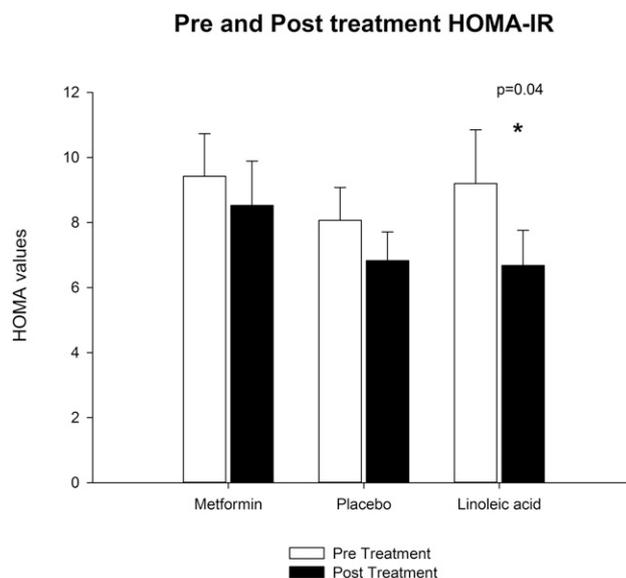
Figure 3. HOMA-IR was significantly lower in the CLA-treated group at study termination compared with initial values. No differences were found in the MET-treated and control groups. The error bars show SEM.

CLA-treated patients) and waist circumference (70%, compared with 60% in PLB and 30% in the CLA group). Total body fat did not improve in any group, but leptin levels significantly decreased in all patients ($P < 0.014$, data not shown).

Racine *et al.* (10) reported a clinical trial in a pediatric population randomly assigned to CLA (3 g/d, *c9,t11-t10-c12*, 50:50) or PLB for 6 months that showed a decrease in total body fat in the CLA group and a significant decrease in HDL cholesterol levels in CLA-treated patients. Our trial demonstrated a significant improvement in HDL cholesterol levels in PLB-treated patients (baseline vs post-intervention, $P = 0.045$). In the CLA group, we noticed a decline in the HDL cholesterol concentration that was not statistically significant when compared with PLB.

One of the limitations of this study was the high rate of participants' withdrawal, as well as the difficulties related to the EHCT, both of which contributed to the final small number of participants, as we did not have enough power for seizing small size effects associated with the treatment.

The strength of this study is supported by its own design. For example, inclusion and, particularly, elimination criteria were strictly applied. Baseline characteristics of participants were similar regarding anthropometric and metabolic condition, particularly those related to subrogated indexes of insulin resistance. Additionally, the main outcome was evaluated by the gold standard EHCT, and the benefits of the overall LIP were evident and similar, regardless of treatment allocation. Although the withdrawal of participants was high in our study, the elimination was random and homogeneous in all groups.

Conclusions

The current study demonstrates the benefits of an LIP and additional effect of CLA over the gold standard EHCT. Lifestyle intervention, independent of any treatment, showed effects on the main outcome variables, specifically weight, height, BMI, waist circumference, surrogate indexes of insulin resistance, and fitness condition, in all of the groups. IRS2 upregulation was evident in CLA-treated patients; this mechanism might be involved in insulin-sensitizing effects on skeletal muscle.

Finally, the incidence of hypertriglyceridemia and hypo-α-lipoproteinemia in CLA-treated patients might be a concern and may be related to the types of CLA isomers used in this study. Further research to evaluate the benefits of different mixtures of CLA isomers may be warranted.

Acknowledgments

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