Ethinyl Estradiol-Cyproterone Acetate Versus Low-Dose Pioglitazone-Flutamide-Metformin for Adolescent Girls with Androgen Excess: Divergent Effects on CD163, TWEAK Receptor, ANGPTL4, and LEPTIN Expression in Subcutaneous Adipose Tissue

Marta Díaz, Matilde R. Chacón, Abel López-Bermejo, Elsa Maymó-Masip, Cristina Salvador, Joan Vendrell, Francis de Zegher, and Lourdes Ibáñez

Endocrinology Unit (M.D., L.I.), and Department of Obstetrics and Gynecology (C.S.), Hospital Sant Joan de Déu, University of Barcelona, 08950 Esplugues, Barcelona, Spain; Hospital Universitari de Tarragona Joan XXIII (M.R.C., E.M.-M., J.V.), Institut d’Investigació Sanitaria Pere Virgili, University Rovira i Virgili, 43007 Tarragona, Spain; Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas (M.D., M.R.C., E.M.-M., J.V., L.I.), Instituto de Salud Carlos III, 28029 Madrid, Spain; Department of Pediatrics (A.L.-B.), Dr. Josep Trueta Hospital, 17007 Girona; Girona Institute for Biomedical Research (A.L.-B.), 17007 Girona, Spain; and Pediatric Endocrinology (F.d.Z.), University Hospital Gasthuisberg, 3000 Leuven, Belgium

Objective: The aim was to compare the effects of a traditional therapy (an oral estroprogestagen) to those of a novel treatment (a low-dose combination of generics) in adolescent girls with androgen excess.

Study Design and Methods: In an open-label trial over 1 yr, 34 adolescents (age, 16 yr; body mass index, 23 kg/m²) with hyperinsulinemic androgen excess and without pregnancy risk were randomized to receive daily ethinyl estradiol-cyproterone acetate (EE-CA; Diane 35 Diario) or a low-dose combination of pioglitazone 7.5 mg/d, flutamide 62.5 mg/d, and metformin 850 mg/d (PioFluMet). Markers of androgen excess, C-reactive protein, high molecular weight adiponectin, lipids, carotid intima media thickness, body composition (absorptiometry), abdominal fat partitioning (magnetic resonance imaging), and gene expression in longitudinal biopsies of sc adipose tissue at the abdominal level (RT-PCR) were assessed at baseline and after 1 yr.

Results: EE-CA and low-dose PioFluMet reduced androgen excess comparably, but had divergent effects on C-reactive protein, high molecular weight adiponectin, lipids, carotid intima media thickness, body composition (absorptiometry), abdominal fat partitioning (magnetic resonance imaging), and gene expression in longitudinal biopsies of sc adipose tissue. All these divergences pointed to a healthier condition on low-dose PioFluMet.

Conclusion: EE-CA and PioFluMet are similarly effective in reversing androgen excess over 1 yr, but low-dose PioFluMet is superior in reversing inflammatory, metabolic, and cardiovascular anomalies that are often associated with androgen excess. (J Clin Endocrinol Metab 97: 3630–3638, 2012)

Abbreviations: AMPK, AMP kinase; ANGPTL4, angiopoietin-like protein 4; BMI, body mass index; cIMT, carotid intima media thickness; CRP, C-reactive protein; Ct, threshold cycle; CV, coefficient of variation; EE-CA, ethinyl estradiol-cyproterone acetate; HMW, high molecular weight; HSD11B1, 11β-hydroxysteroid dehydrogenase type 1; LDL, low-density lipoprotein; LEPTIN, leptin; LPL, lipoprotein lipase; MRI, magnetic resonance imaging; NF-κB, nuclear factor-κB; PCOS, polycystic ovary syndrome; PioFluMet, pioglitazone-flutamide-metformin; PPARγ, peroxisome proliferator-activated receptor γ.
In adolescent girls with ovarian androgen excess, there are essentially two therapeutic strategies to revert the signs and symptoms associated with hyperandrogenism, such as hirsutism, acne, and irregular menses. The classic approach is an ovary-silencing therapy with oral contraceptives containing different combinations of estroprogestagens; among them, ethinyl estradiol-cyproterone acetate (EE-CA) is commonly used for its purported antiandrogenic properties (1, 2). An alternative approach is to correct the underpinning metabolic abnormalities—frequently including hyperinsulinemic insulin resistance—thereby normalizing ovarian function and attenuating the risk for subsequent diabetes and cardiovascular disease (3, 4). In the latter approach, insulin-sensitizing agents such as metformin, have been used alone (3), or in a low-dose combination with flutamide, or with both flutamide and pioglitazone (PioFluMet); such combinations were recently shown to have beneficial effects on body composition, on markers of low-grade inflammation, and on circulating adipokines in adolescents and young women with ovarian androgen excess (5, 6).

Adipose tissue dysfunction in hyperandrogenic girls—who combine enlarged adipocytes, hyperadiponectinemia, and decreased lipoprotein lipase (LPL) activity—may be a key factor in the pathogenesis of insulin resistance (7). Gene expression studies in sc and visceral adipose tissue of women with androgen excess point to a dysregulation of functional pathways involved in insulin signaling and resistance, adipogenesis, lipid metabolism, inflammation, immune function, and oxidative stress (8–11). So far, these studies have included only a few adolescents and/or nonobese patients (12) and have not addressed the potential effects of pharmacological intervention.

In a first head-to-head study over 6 months, EE-CA and low-dose PioFluMet were found to have comparable effects on hyperandrogenemia in girls with androgen excess, but divergent effects on cardiovascular health markers, adipokines, visceral fat, and low-grade inflammation—all these divergences favoring treatment with PioFluMet (13). Here, we report the results over 12 months together with a first comparison of gene expression patterns in consecutive biopsies of sc adipose tissue. We hypothesized that the two treatments would also have divergent effects on gene expression in adipose tissue, and accordingly, we assessed the longitudinal changes in gene expression within subgroups (0–12 months) and the differences in 0- to 12-month changes between subgroups. For the purpose of the study, we elected to study genes encoding transcription factors and other proteins involved in glucose and lipid metabolism and in inflammatory pathways.

Subjects and Methods

Study population and design

The study population consisted of 34 girls with hyperinsulinemic androgen excess [age, 16 yr; range, 14–17 yr; body mass index (BMI), 23 kg/m²; range, 19–29 kg/m²]; all of them were at least 2 yr beyond menarche. The girls were recruited among patients consecutively seen in the Adolescent Endocrinology Unit of Sant Joan University Hospital, Barcelona, Spain, between January and November 2010. The recruitment procedure has been previously reported in detail (13).

As described (13), the inclusion criteria were: 1) hyperinsulinemia, defined as fasting insulin above 15 μU/ml, and/or a peak insulin above 150 μU/ml, and/or mean insulin greater than 84 μU/ml on a 2-h oral glucose tolerance test (14); 2) clinical and biochemical androgen excess, as defined by the presence of hirsutism (score above 8 in the Ferriman and Gallwey scale), amenorrhea (absence of menses for more than 3 months), or oligomenorrhea (menstrual cycles of >45 d), and high serum levels of androstenedione and/or testosterone in the follicular phase of the cycle (d 3–7) or after 2 months of amenorrhea.

Exclusion criteria were: pregnancy risk at study start or on follow-up, evidence of anemia, thyroid dysfunction, bleeding disorder, Cushing syndrome, hyperprolactinemia, glucose intolerance, diabetes mellitus, late-onset adrenal hyperplasia due to 21-hydroxylase deficiency (17-hydroxyprogesterone levels ≥200 ng/dl in the follicular phase of the cycle or after 2 months of amenorrhea), abnormal electrolytes or abnormal liver or kidney function, and use of medication affecting gonadal or adrenal function, or carbohydrate or lipid metabolism.

Randomization was performed with the SealedEnvelope program (Sealed Envelope Ltd., London, UK) (http://www.SealedEnvelope.com), using random permuted blocks with strata for age and BMI (13). The girls were randomly assigned to receive daily, at dinner time, either Diane 35 Diario (Bayer-Schering, Madrid, Spain; 35 μg ethinyl estradiol plus 2 mg cyproterone acetate for 21 of 28 d and placebo for 7 of 28 d) or PioFluMet in low-dose [7.5 mg pioglitazone (one half of a 15-mg tablet of Actos); Takeda, Madrid, Spain]; 62.5 mg flutamide (one quarter of a 250-mg tablet of Flutamida; Cifna, Navarra, Spain), and 850 mg metformin (one full 850-mg tablet of Metformina; Sandoz, Barcelona, Spain), as reported (13).

Clinical and endocrine-metabolic variables, carotid intima media thickness (cIMT), body composition, and abdominal fat partitioning were all assessed at study start (0 months) and after 12 months. Adipose tissue biopsies were performed at baseline and after 12 months of treatment.

The study was registered as ISRCTN45546616 and was conducted in Barcelona, without support from the pharmaceutical industry, after approval by the Institutional Review Board of Sant Joan de Déu University Hospital, after written consent by the parents, and after assent by each of the study girls. None of the 12-month results have previously been reported.

Clinical and endocrine-metabolic assessment

The same investigator (L.I.) measured weight and height (Harpenden stadiometer), derived BMI, and scored hirsutism (Ferriman-Gallwey) and acne (Leeds grading scale).

Baseline assessments were performed in the follicular phase (d 3–7) or after 2 months of amenorrhea. Fasting blood glucose,
serum insulin, lipid profile, SHBG androgens, C-reactive protein (CRP), and high molecular weight (HMW) adiponectin were assessed together with blood count and a screening of liver and kidney function.

Serum glucose, immunoreactive insulin, SHBG, and testosterone and serum lipids were assayed as described (13). CRP was measured with a highly sensitive method (Architect c8000; Abbott, Wiesbaden, Germany) with intra- and interassay coefficients of variation (CV) of less than 2% and a detection limit of 0.1 mg/liter. HMW adiponectin was assessed with an ELISA kit (13); the intra- and interassay CV were 2.4 and 8%, respectively. Serum samples were stored at −20 C until assay.

Carotid intima media thickness

Longitudinal ultrasound scans of the carotid arteries were obtained by the same investigator (blinded to treatment allocation), with high-resolution equipment (Acuson Sequoia 512 SHA; Medisales, Los Alamitos, CA) (13). The mean values obtained on the left and right sides were averaged; the intraobserver CV was less than 10%.

Body composition and abdominal fat partitioning

Body composition was assessed by dual-energy x-ray absorptiometry with a Lunar Prodigy and Lunar software (version 3.4/3.5; GE Lunar Corp., Madison, WI); absolute whole body fat and lean mass (kilograms), as well as fat content in the abdominal region were assessed (13, 15).

The sc vs. visceral partitioning of abdominal fat was assessed by magnetic resonance imaging (MRI) using a multiple-slice MRI 1.5 Tesla scan (Signa LX Echo Speed Plus Excite; General Electric, Milwaukee, WI); MRI was also used to assess intradepartmental fat content (13, 15). All scans were performed by the same operator, and all images were analyzed by the same radiologist (both blinded to treatment allocation).

Adipose tissue biopsy

Longitudinal adipose tissue gene expression studies were performed in 31 of 34 girls (n = 14 in the Diane subgroup, and n = 17 in the PioFluMet subgroup). In one girl, no biopsy was performed because she had a history of hypertrophic scars; two other girls consented in having the baseline biopsy but declined to undergo a second biopsy at 12 months.

Subcutaneous adipose tissue samples were obtained through a 5- to 7-mm cold scalpel incision about 1 cm below the umbilicus, under local anesthesia (2% mepivacaine). An anesthetic cream (Emla; AstraZeneca, Madrid, Spain) was applied in the periumbilical area in the previous 5–7 h to further minimize the discomfort of the procedure. The samples were immediately frozen in liquid nitrogen and stored until analysis.

Quantitative RT-PCR

Between 10 and 50 mg of frozen sc white adipose tissue was homogenized using Polytron benchtop homogenizer (Kinetica Ag, Littau-Luzern, Switzerland) for RNA extraction. Total RNA was isolated using Tripure reagent (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s instructions. The quantity and quality of isolated RNA were determined with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), respectively.

Between 0.2 and 1 μg of total RNA was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents (Applied Biosystems, Foster City, CA) and subsequently diluted with nuclelease-free water (Sigma, St. Louis, MO) to 20 ng/μl cDNA. The gene amplifications were assessed using specific pairs of primers; the assay IDs for each predesigned probe are listed in Table 1.

Real-time quantitative PCR was performed with duplicates on a 7900HT Fast Real-Time PCR System using commercial Taqman Assays (Applied Biosystems). SDS software 2.3 and RQ Manager 1.2 (Applied Biosystems) were used to analyze the results with the comparative threshold cycle (Ct) method (2^ΔΔCt) (16). Ct values for each sample were normalized with an optimal reference gene (GAPDH; Hs03929097), after testing three additional housekeeping genes: β-actin, cyclophilin, and 18s RNA. GAPDH was selected based on its stable expression in all samples and conditions analyzed. All data were expressed as an n-fold difference relative to a calibrator (mixing T = 0 and T = 12 from one sample of each treatment arm).

The relative expression results of the selected genes are reported in Table 1.

Statistical analyses

Statistical analyses were performed with SPSS 12.0 (SPSS, Chicago, IL). For uniformity, results are expressed as mean ± SEM. Results with non-Gaussian distributions were mathematically transformed to improve symmetry before statistical analysis. The effects of each treatment over time were assessed by using unpaired Student’s t test. Two-way ANOVA followed by Bonferroni post hoc comparison test (Prism 5 software; GraphPad Software, Inc., San Diego, CA) was used to assess the simultaneous interaction of treatment and time on gene expression changes. Associations between continuous variables were sought by correlation analyses. P < 0.05 was considered statistically significant.

Results

Endocrinology, body composition, and abdominal fat partitioning

EE-CA and PioFluMet had similarly normalizing effects on clinical and biochemical markers of androgen excess. Divergent effects were observed on fasting insulin, total and low-density lipoprotein (LDL)-cholesterol, CRP, HMW adiponectin, cIMT, lean mass, and abdominal and visceral fat (Table 2 and Fig. 1), all in favor of PioFluMet. Liver and renal function tests remained within normal limits (at 12 months, for EE-CA and PioFluMet subgroups: alanine aminotransferase, 14 ± 1 and 15 ± 2 IU/liter; aspartate aminotransferase, 16 ± 1 and 17 ± 1 IU/liter; creatinine, 0.68 ± 0.01 and 0.67 ± 0.01 mg/dl).

Gene expression analysis: comparisons within subgroups

Gene expression analysis at baseline was comparable between subgroups for all assessed genes (Table 1).
The longitudinal changes within subgroups (0–12 months) disclosed an up-regulation of 78 kDa glucose-regulated protein precursor (HSPA5), of serine-threonine kinase 11 (STK11), and of TNF-like weak inducer of apoptosis (TWEAK) receptor [also named fibroblast growth factor-inducible 14 (Fn14)] mRNA expression in EE-CA-treated girls (Table 1 and Fig. 2A), and a down-regulation in the mRNA expression of peroxisome proliferator-activated receptor γ (PPARγ), 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1), and leptin (LEP) in the PioFluMet-treated girls (Table 1 and Fig. 2B) (all P < 0.05 to P < 0.01 vs. baseline). In the latter subgroup, STK11 expression tended to increase after 12 months.

## Gene expression analysis: comparisons between subgroups

The comparison of gene expression profiles between treatments after 12 months disclosed an up-regulation of CD163, TWEAK receptor, LEP, and of angiotensin-like protein 4 (ANGPTL4) in EE-CA-treated girls (P < 0.05 to P < 0.01 vs. PioFluMet-treated girls; Table 1 and Fig. 2C).

### Correlations

The up-regulation in CD163 expression in EE-CA-treated girls was associated with the increase in visceral fat (r = 0.59; P < 0.005) and the decrease in circulating HMW adiponectin (r = 0.41; P = 0.04), whereas the up-
**Table 2.** Data from adolescent girls with androgen excess who were randomized to receive EE-CA (n = 17) or low-dose PIOFLUMET (n = 17) for 12 months

<table>
<thead>
<tr>
<th></th>
<th>EE-CA Baseline 12 months</th>
<th>Δ 0–12 months</th>
<th>Low-dose PIOFLUMET Baseline 12 months</th>
<th>Δ 0–12 months</th>
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<tbody>
<tr>
<td>Age (yr)</td>
<td>15.9 ± 0.3</td>
<td>23.9 ± 0.6</td>
<td>0.8 ± 0.2</td>
<td>16.5 ± 0.3</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.0 ± 0.8</td>
<td>9.1 ± 0.6d</td>
<td>−4.5 ± 0.7</td>
<td>14.0 ± 0.9</td>
</tr>
<tr>
<td>Hirsutism score</td>
<td>13.5 ± 0.9</td>
<td>9.1 ± 0.6d</td>
<td>−4.5 ± 0.7</td>
<td>14.0 ± 0.9</td>
</tr>
<tr>
<td>Acne score</td>
<td>2.2 ± 0.2</td>
<td>1.1 ± 0.1d</td>
<td>−1.1 ± 0.2</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>Endogenously regular cycles</td>
<td>35%</td>
<td>−35%</td>
<td></td>
<td>41%</td>
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<tr>
<td>SHBG (nmol/liter)</td>
<td>23 ± 3</td>
<td>162 ± 8d</td>
<td>139 ± 8</td>
<td>28 ± 3</td>
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<tr>
<td>Testosterone (ng/dl)</td>
<td>58 ± 7</td>
<td>30 ± 3d</td>
<td>−27 ± 6</td>
<td>63 ± 7</td>
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<td>Fasting insulin (µU/ml)*</td>
<td>9.7 ± 1.1</td>
<td>−4.4 ± 1.6</td>
<td></td>
<td>10.2 ± 1.9</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dl)*</td>
<td>81 ± 3</td>
<td>24 ± 6</td>
<td></td>
<td>80 ± 5</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)*</td>
<td>50 ± 2</td>
<td>14 ± 2</td>
<td></td>
<td>56 ± 3</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)*</td>
<td>89 ± 20</td>
<td>43 ± 17</td>
<td></td>
<td>66 ± 11</td>
</tr>
<tr>
<td>CRP (mg/liter)*</td>
<td>0.9 ± 0.2</td>
<td>1.7 ± 0.4</td>
<td>−0.7 ± 0.2</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>HMW adiponectin (mg/liter)*</td>
<td>15 ± 3</td>
<td>7 ± 1*</td>
<td>−8 ± 2</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>cIMT (mm)</td>
<td>0.35 ± 0.01</td>
<td>0.37 ± 0.01c</td>
<td>0.02 ± 0.01</td>
<td>0.35 ± 0.01</td>
</tr>
<tr>
<td>Absorptiometry</td>
<td>BMD (g/cm²)</td>
<td>1.13 ± 0.03</td>
<td>1.14 ± 0.02</td>
<td>1.17 ± 0.02</td>
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<tr>
<td></td>
<td>Lean mass (kg)</td>
<td>35.2 ± 0.7</td>
<td>34.0 ± 0.8e</td>
<td>37.4 ± 0.7</td>
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<tr>
<td></td>
<td>Fat mass (kg)</td>
<td>19.9 ± 1.9</td>
<td>22.0 ± 1.5d</td>
<td>18.7 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Abdominal fat (kg)</td>
<td>5.3 ± 0.4</td>
<td>5.9 ± 0.4f</td>
<td>5.3 ± 0.3</td>
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<tr>
<td></td>
<td>Lean mass minus fat mass</td>
<td>15.2 ± 0.7</td>
<td>12.0 ± 0.5c</td>
<td>18.7 ± 0.5</td>
</tr>
<tr>
<td>MRI</td>
<td>Abdominal fat (cm²)</td>
<td>163 ± 17</td>
<td>196 ± 19c</td>
<td>164 ± 16</td>
</tr>
<tr>
<td></td>
<td>Visceral fat (cm²)</td>
<td>32 ± 2</td>
<td>37 ± 2b</td>
<td>36 ± 3</td>
</tr>
<tr>
<td></td>
<td>Intrahepatic lipid content (%)</td>
<td>16.9 ± 1.6</td>
<td>17.1 ± 1.4</td>
<td>15.1 ± 1.5</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. HDL, High-density lipoprotein; BMD, bone mineral density.

1 100% of these girls had menses with an invariable interval of 28 d, occurring during the intake of placebo pills for 7 of 28 d.

* Indicative values in healthy adolescents (n = 18; age, 16.9 ± 0.3 yr; BMI, 21.1 ± 0.6 kg/m²; fasting insulin, 3.0 ± 0.3 µU/ml; LDL-cholesterol, 81 ± 5 mg/dl; HDL-cholesterol, 54 ± 2 mg/dl; triglycerides, 54 ± 4 mg/dl; CRP, 0.7 ± 0.2 mg/liter; HMW adiponectin, 13 ± 2 mg/liter.

a No significant differences between randomized subgroups at start.

b p < 0.05; c p ≤ 0.01; d p < 0.001, within subgroups for 0- to 12-month change (Δ).

e p < 0.05; f p ≤ 0.01; g p = 0.001 between subgroups for 0- to 12-month change (Δ).

regulation in TWEAK receptor was related to the increase in CRP (r = 0.49; P = 0.01) after 12 months (Supplemental Fig. 1).

**Discussion**

**Main findings**

The present study indicates that EE-CA and PIOFLUMET are similarly effective in the reversal of clinical and biochemical hyperandrogenism over at least 12 months but have divergent effects on markers of metabolic health and on the adipose-tissue expression of genes such as CD163, TWEAK receptor, LEP, and ANGPTL4, respectively related to macrophage activation, inflammation, fat accretion, and lipoprotein metabolism. Of note, the divergences observed in cIMT—an established cardiovascular risk marker—after 6 months were maintained after 12 months. All divergences point to a healthier condition in adolescents receiving PIOFLUMET.

**CD163, macrophage activation, and inflammation**

CD163 is a member of the scavenger receptor cysteine-rich superfamily, is almost exclusively expressed on monocytes and macrophages, and is closely related to macrophage activation (17). Typically, this marker is associated with the M2 macrophage subpopulation involved in the down-regulatory phase of the inflammatory process. Recently, increased CD163 expression was found in crown-like structures within sc adipose tissue of obese individuals, reflecting macrophage infiltration and inflammation and suggesting a compensatory activation of the immunosuppressive pathways in states of low-grade inflammation (18). The increase in CD163 expression was correlated with visceral fat deposition (independent of adiposity) and associated with increased levels of proinflammatory cytokines whose expression is regulated by the nuclear factor-κB (NF-κB) stress pathway, which may impair insulin signaling and result in insulin resistance and ectopic lipid accumulation (18, 19). The CD163 up-reg
ulation in the EE-CA-treated girls in parallel with the increase in the mRNA levels of proinflammatory genes such as TWEAK receptor or LEP supports the notion that prolonged estroprogestagen intake may enhance the state of low-grade inflammation characteristic of ovarian androgen excess, and as a result, the CD163 compensatory activation process mentioned above may be operative.

**TWEAK receptor and CD163 interaction: effects of metformin and adiponectin**

CD163 may also bind TWEAK—a proinflammatory cytokine in adipocytes—in pathological conditions, serving as an alternate receptor for TWEAK in cells lacking its receptor (20). The proinflammatory actions and increased expression of both TWEAK and its receptor (Fn14) in adipocytes have been shown to be mediated by NF-κB (21, 22) in humans. In girls receiving EE-CA, the combined increase in CD163 and TWEAK receptor expression was closely related to the changes in visceral fat, the increase in CRP—a marker of low-grade inflammation—and the decrease in HMW adiponectin after 12 months. These data suggest that estroprogestagens, when given in supraphysiological doses, may deregulate the CD163-TWEAK system, resulting in a stimulation of the NF-κB stress pathway, which in turn may enhance the already existing metabolic and body composition abnormalities. In addition, adiponectin and metformin have been shown recently to down-regulate cellular CD163 in vitro, probably through AMP kinase (AMPK) activation, resulting in a suppression of inflammation and in a modulation of cytokine signaling pathways (23, 24). This mechanism may also have influenced the relative difference in CD163 expression after 12 months in the two subgroups, with exposing influences of metformin (accounting for CD163 down-regulation in the PioFluMet girls) and decreasing levels of HMW adiponectin (resulting in CD163 up-regulation in EE-CA girls). Notwithstanding, the changes in adipose tissue AMPK activity in vitro, as described in diabetic men treated with metformin (25), are not necessarily paralleled by changes in gene expression in vivo. To our knowledge, no studies have been performed in polycystic ovary syndrome (PCOS) women, assessing the effects of metformin on CD163 and/or AMPK expression/activity.

**ANGPTL4 and lipids**

The up-regulated ANGPTL4 expression in EE-CA-treated girls may have contributed to the rise of circulating LDL-cholesterol and triglyceride levels observed in these patients after 12 months. Indeed, ANGPTL4 is preferentially expressed in adipose tissue, wherein it serves as a regulator of lipolysis; in peripheral tissues, ANGPTL4 regulates fatty acid uptake through inhibition of LPL by promoting conversion of active LPL dimers into inactive monomers (26, 27). Genetic knockout of ANGPTL4 in mice protects against development of atherosclerosis and suppresses the ability of macrophages to become foam cells in vitro (28); in contrast, ANGPTL4 overexpression results in hypertriglyceridermia and peripheral insulin resistance (26). In diabetic mice, metformin down-regulates ANGPTL4 expression in liver and in adipose tissue (28), whereas systemic PPARγ stimulation facilitates adipose tissue ANGPTL4 secretion, resulting in angiogenesis and endothelial cell growth necessary for adipose tissue expansion (29). It is tempting to speculate that in the PioFluMet-treated girls, the joint effects of metformin and low-dose pioglitazone may have accounted for a relatively lower expression of ANGPTL4, supporting the notion that low-dose pioglitazone acts as inhibitor of CDK5-mediated phosphorylation of PPARγ rather than as a PPARγ activator (30). Interestingly, in the PioFluMet girls, PPARγ was significantly down-regulated after 12 months, suggesting that this combination may indeed tone down adipocyte differentiation activity.

**LEP, adiposity, and inflammation**

EE-CA-treated girls also showed increased LEP mRNA levels after 12 months, as compared with the PioFluMet girls where a significant down-regulation of LEP was ob-
served after 12 months. Previous studies have revealed that
leptin (LEP) expression in hyperandrogenic women is more
related to adiposity than to androgen excess (10, 31); how-
ever, most of these studies have been performed in obese
women and have failed to clarify the relationship between
LEP expression and the androgen excess phenotype (32,
33). It is well known that leptin may alter insulin action in
isolated adipocytes and that it promotes glucose and fatty
acid oxidation and lipolysis (34). These observations from
in vitro and in vivo studies suggest that leptin promotes
energy dissipation and decreases lipid deposition in ad-
ipose tissue. LEP overexpression in EE-CA-treated girls
may reflect the increase in visceral fat and might be a
feedback mechanism to compensate for this unfavor-
able pattern. Finally, the increase in LEP expression
could be part of the inflammatory response, reflecting
an unfavorable inflammatory profile in the adipose tis-

Longitudinal gene expression changes within
subgroups

The potential implications of the gene expression
changes occurring only within subgroups deserve further
consideration. For example, increased expression of
HSD11β1 has been reported in insulin-resistant states,
including in obesity and androgen excess (9, 36). In hy-
perandrogenic women, the increase in HSD11β1 expres-
sion has also been related to increased upper body fat
distribution, even in the absence of obesity (37). The
down-regulation in HSD11β1 expression after PioFluMet
coincided with a decrease in fasting insulin and in abdomi-

FIG. 2. Results of gene expression analyses in sc adipose tissue of adolescent girls with androgen excess who were randomized to receive EE-CA
(n = 14) or low-dose PioFluMet (n = 17) for 12 months. A and B, Histograms show mean and SEM results for 0- to 12-month changes in gene
expression for EE-CA-treated (A) and PioFluMet-treated (B) girls. C, The between-treatment differences in 0- to 12-month changes in gene
expression. Significant within- and between-treatment differences in gene expression are marked with asterisks.

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metformin therapy in hyperinsulinemic girls with androgen excess (38), low-dose flutamide could henceforth be viewed as a “metformin sensitizer” in the adipose tissue of such girls.

Finally, the increase in the expression of HSPA5—a resident chaperone within the endoplasmic reticulum—may reflect enhanced endoplasmic reticulum stress in adipocytes after EE-CA intervention; indeed, HSPA5 up-regulation has been linked to apoptosis and β-cell dysfunction in both animal and human models (39, 40).

Strengths and weaknesses
The present study appears to be the first to assess gene expression in sc adipose tissue of nonobese PCOS adolescents. The longitudinal design allowed us to detect divergent effects of EE-CA and low-dose PioFluMet on gene regulation in adipose tissue and to disclose a parallelism between changes in phenotype and in adipose-tissue gene expression.

The main limitation of the study is the scarce amount of adipose tissue obtained with a minimal scalpel incision; this limitation precluded the assessment of adipose tissue morphology and also impeded the study of protein expression.

Conclusion
In conclusion, EE-CA and PioFluMet were similarly effective in reverting hyperandrogenism in nonobese PCOS adolescents but had divergent effects on the cardio- metabolic phenotype, including on cIMT, paralleled by differences in the expression of genes related to macrophage activation, inflammation, lipoprotein metabolism, and fat accretion, all the divergences being in favor of low-dose PioFluMet.

Further exploration of low-dose PioFluMet seems warranted, not only to strengthen its safety side over the longer term and in larger cohorts, but also to verify further whether the efficacy of this low-dose combination holds enough potential to allow it to become a first-choice therapy for the majority of young adolescents with androgen excess, namely those who are not at pregnancy risk and who are nowadays nevertheless exposed to supraphysiological doses of estroprogestagens.

Acknowledgments
We thank Luis del Río, M.D., and Silvana di Gregorio, M.D., from Centro Médico CETIR, Barcelona, Spain, for performing the absorptiometry and MRI measurements. We are grateful to Giorgia Sebastiani for performing the carotid ultrasound assessments.

Address all correspondence and requests for reprints to: Lourdes Ibáñez, M.D., Ph.D., Hospital Sant Joan de Déu, University of Barcelona, Passeig de Sant Joan de Déu, 2, 08950 Esplugues, Barcelona, Spain. E-mail: libanez@hsjdbcn.org.

This study was supported by grants from the Ministerio de Ciencia e Innovación, Instituto de Salud Carlos III, Madrid, Spain (PI09/90444, PI11/00049, and PI11/00085). M.R.C. is supported by a fellowship from the Fondo de Investigación Sanitaria (FIS) “Miguel Servet” CP 06/00119. F.d.Z. is a Clinical Investigator supported by the Clinical Research Council of the Leuven University Hospitals, and by the University of Leuven (OT-04-35). A.L.-B. is a Clinical Investigator of the I3 Fund for Scientific Research (Ministry of Science and Innovation, Spain).


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