High neutrophil count in girls and women with hyperinsulinaemic hyperandrogenism: normalization with metformin and flutamide overcomes the aggravation by oral contraception

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BACKGROUND: The endocrine hallmark of polycystic ovary syndrome (PCOS) is hyperinsulinaemic hyperandrogenism; another facet of PCOS is low-grade inflammation. METHODS: In adolescents and young women with hyperinsulinaemic hyperandrogenism (n = 118; mean age 16 years, body mass index 22 kg/m²), we analysed whether the PCOS-associated rise in leukocyte count is already detectable at young age and, if so, whether such elevation is lowered by metformin, flutamide-metformin, oral contraception (OC), or their combination.

RESULTS: Leukocyte count (×1000/mm³) in patients was high versus controls (7.5 ± 0.1 versus 6.4 ± 0.1; P < 0.001) due to a rise in neutrophils (4.2 ± 0.1 versus 3.0 ± 0.1; P < 0.001). Randomized studies at mean ages of 12.5 years (n = 24) and 15.2 years (n = 33) demonstrated normalizing effects of metformin (850 mg/day; P < 0.001) and, respectively, metformin plus flutamide (62.5 mg/day) on neutrophil counts; in young women (18.3 years; n = 41), the neutrophil count rose further on OC monotherapy (P = 0.003), but normalized on the same OC plus flutamide-metformin (P < 0.001 versus OC alone). CONCLUSIONS: (i) A high leukocyte count is already present in girls with hyperinsulinaemic hyperandrogenism, and this is due to a raised neutrophil count; (ii) this hyperneutrophilia is attenuated by metformin or flutamide-metformin, and is amplified by OC monotherapy; (iii) if these treatments are combined, the normalizing effect of flutamide-metformin overcomes the OC effect on neutrophil count.

Key words: flutamide/leukocytosis/metformin/oral contraception/ovarian hyperandrogenism

Introduction

Polycystic ovarian syndrome (PCOS), a variable constellation of anovulatory hyperandrogenism with hyperinsulinaemia and/or dyslipidaemia, is the most frequent endocrine disorder of young women (Asunció et al., 2000; Dunaif and Thomas, 2000; Baumann and Rosenfield, 2002).

One of the multiple facets of PCOS is a state of low-grade inflammation, as judged by moderate elevations of circulating markers such as C-reactive protein (CRP), interleukin-6 (IL-6) and leukocyte count (Kelly et al., 2001; Morin-Papunen et al., 2003a; Bouman et al., 2004; Ibáñez et al., 2004a; Tarkun et al., 2004; Orio et al., 2005). This chronic, low-grade inflammatory state has been linked to the degree of insulin resistance and to the early development of atherosclerosis (Tarkun et al., 2004; Orio et al., 2005). Treatment effects on the leukocyte count are not available but, using CRP and IL-6 as markers, metformin has been shown to attenuate the pro-inflammatory state, while oral contraceptives (OC) seem to aggravate it, even if they contain cyproterone acetate or drospirenone as progestagen (Morin-Papunen et al., 2003a; Ibáñez et al., 2004a; Ibáñez and de Zegher, 2004a).

Flutamide–metformin plus ethinylestradiol–drospirenone has been developed as a combined therapy capable of reverting the hyperinsulinaemic hyperandrogenism, anovulation, dyslipidaemia, adipocytokine imbalance and body adiposity of PCOS toward normal, and that may therefore improve the long-term prognosis of women with PCOS (Ibáñez and de Zegher, 2003, 2004a; Ibáñez et al., 2003a); low-dose flutamide does not appear to be hepatotoxic (Ibáñez et al., 2005); both metformin and flutamide play a pivotal role in the efficacy (Ibáñez et al., 2002a, 2004b; Ibáñez and de Zegher, 2005) and, for safety reasons, this duo has been complemented by an OC, a drospirenone OC being abdominally more lipolytic than a gestodene OC (Ibáñez and de Zegher, 2004b). In the early stages of PCOS development (age 8–14 years), metformin in monotherapy lowers
circulating IL-6 effectively, provided there is no associated obesity (Ibañez et al., 2004a).

We have now analysed whether the PCOS-associated rise in leukocyte count, as reported in women aged ~25 years (Orio et al., 2005), is already detectable at a younger age and, if so, whether such elevation is lowered by treatment with metformin, flutamide–metformin, OC or a combination of these.

Study population and methods

Study population and ethics

Prompted by the report of a raised leukocyte count in women with PCOS (Orio et al., 2005), we analysed the unpublished blood count results from our recent studies which explored new treatments in girls, adolescents and young women with established or incipient PCOS, but which focused primarily on other efficacy and safety indices. Incipient PCOS was defined as a condition in which girls already present the endocrine–metabolic abnormalities of PCOS, but do not yet present hirsutism (Ferriman–Galway score < 8) or menstrual disturbances (Ferriman and Gallwey, 1961; Ibañez et al., 2002b, 2004c). Table I lists the baseline characteristics of the total study population (n = 118). Table II displays the treatment options that were compared in randomized studies (Ibañez and de Zegher, 2003, 2004a; Ibañez et al., 2003, 2004c) including a total of 24 non-obese girls with incipient PCOS, and 94 non-obese adolescents or young women with PCOS.

All studies were conducted in Barcelona, none being supported by the pharmaceutical industry. All studies were approved by the Institutional Review Board of Sant Joan University Hospital; informed consent was obtained from parents or young women, and assent from minors.

Table I. Baseline data in non-obese girls, adolescents and young women with hyperinsulinaemic hyperandrogenism, and in healthy controls matched for age, height and weight

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 78)</td>
<td>(n = 118)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>15.7 ± 0.3</td>
<td>15.9 ± 0.2</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>21.6 ± 0.6</td>
<td>21.8 ± 0.2</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>13.1 ± 0.1</td>
<td>13.3 ± 0.1</td>
</tr>
<tr>
<td>Leukocytes (×1000/mm³)</td>
<td>6.4 ± 0.1</td>
<td>7.5 ± 0.1</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>3.0 ± 0.1</td>
<td>4.2 ± 0.1</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>2.6 ± 0.1</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>4.5 ± 0.1</td>
<td>4.8 ± 0.1</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>80 ± 4</td>
<td>96 ± 3</td>
</tr>
<tr>
<td>SHBG (nmol/l)</td>
<td>67 ± 4</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>1.0 ± 0.1</td>
<td>3.7 ± 0.1</td>
</tr>
<tr>
<td>Androstenedione (nmol/l)</td>
<td>4.2 ± 0.5</td>
<td>10.3 ± 0.3</td>
</tr>
<tr>
<td>DHEA-S (μmol/l)</td>
<td>3.6 ± 0.4</td>
<td>6.8 ± 0.2</td>
</tr>
</tbody>
</table>

Values are mean ± SEM.

*p = 34 for endocrine–metabolic variables.

p < 0.01.

p < 0.001.

p < 0.0001 versus controls.

SHBG = sex hormone-binding globulin; DHEA-S = dehydroepiandrosterone-sulphate.

Each study population has been reported in detail (Ibañez et al., 2003, 2004a,b,c; Ibañez and de Zegher, 2003, 2004a,b,c).

Common inclusion criteria were: (i) post-menarcheal status; (ii) hyperinsulinaemia on a standard 2 h oral glucose tolerance test (oGTT), defined as peak serum insulin levels > 150 mU/ml and/or mean serum insulin > 84 mU/ml (Ibañez et al., 1997; Vidal-Puig and Moller, 1997); (iii) excessive 17-hydroxyprogesterone (17-OHP) response (> 160 ng/dl) to GnRH agonist (leuprolide acetate, 500 mg s.c.; Abbott, Madrid, Spain) (Ibañez et al., 2002b,c); (iv) body mass index (BMI) < 26 kg/m².

Specific inclusion criteria for Study 1 [untreated versus metformin; Table II (Ibañez et al., 2004c)] were: (i) menarche 6–12 months before study start; (ii) birthweight for gestational age below −1.5 SDS (this corresponds to a birthweight below ~2.7 kg in term Catalan girls); (iii) history of precocious pubarche [presence of pubic hair before the age of 8 years (Ibañez et al., 2004a,c), because the combination of these criteria is known to confer a high risk for progression to full-spectrum PCOS in adolescence (Ibañez et al., 1998, 2001a, 2004a,c).

Specific inclusion criteria for Studies 2 and 3 [untreated versus flutamide plus metformin; Table II (Ibañez et al., 2003)], and Studies 4 and 5 [OC versus OC plus flutamide–metformin; Table II (Ibañez et al., 2003, 2004a): (i) menarche ≥2 years before study start; (ii) hirsutism (Ferriman–Galway score ≥8), amenorrhoea (no menses for >3 months), or oligomenorrhoea (intermenstrual phase of >45 days); (iii) hyperandrogenaemia [elevated serum androstenedione, total testosterone, or free androgen index [testosterone × 100/sex hormone binding globulin (SHBG)]] (Ibañez et al., 1997, 2002c).

Common exclusion criteria were: evidence for thyroid dysfunction, Cushing syndrome or hyperprolactinaemia; glucose intolerance (Expert Committe on the Diagnosis and Classification of Diabetes Mellitus, 1997), family or personal history of diabetes mellitus; late-onset congenital adrenal hyperplasia (New et al., 1983; Sakkal-Alkadour et al., 1996); use of medication known to affect gonadal or adrenal function, or carbohydrate–lipid metabolism; anaemia or serum electrolyte anomalies; smoking; abnormal results in screening tests for liver and kidney function; acute inflammatory processes, as identified by history or physical examination.

Specific exclusion criteria for Study 1 [untreated versus metformin] were: the presence of hirsutism or menstrual disturbances at start of study.

Study design

All studies were open-labelled and compared 3 month treatment effects between randomized groups. Table II lists the compared treatments, the medication doses, and the age groups.

Haemoglobin, leukocyte count and endocrine–metabolic assessment

Fasting blood glucose, haemoglobin (Hb) and leukocyte count were determined at 0–3 months, within 2 h after
venipuncture; among the other assessed variables were serum insulin, lipid profile, SHBG, testosterone, and indices of hepatic and renal function.

Baseline assessments were performed in the follicular phase (day 3–7) or after 2 months of amenorrhoea. Haemoglobin, leukocyte count and hormonal levels were compared to healthy post-menarcheal females of similar age and BMI; this control group was composed of girls with short–normal stature (percentile range 10–25), and adolescents and young women seen for screening purposes at Barcelona Hospital. Control girls received no medication for 3 months, and presented no evidence of thyroid dysfunction, an acute inflammatory disorder, liver or kidney dysfunction.

Assays and statistics
Haemoglobin and leukocyte count were assessed by an automatic cell counter (ABX Pentra 120; ABX Diagnostics, Montpellier, France) calibrated with an ABX Minotrol 16 (ABX Diagnostics); the intra-assay coefficient of variation (CV) determined on five replicates of each leukocyte measurement was \( \leq 1\% \). Glucose was measured by the glucose oxidase method. Immunoreactive insulin was assayed by IMX (Abbott, Santa Clara, CA, USA); intra- and inter-assay CV were 4.7 and 7.2%. Serum testosterone, androstenedione, 17-OHP and SHBG were assayed as described (Ibañez and de Zegher 2003, 2004a; Ibañez et al., 2003, 2004c). Samples for hormonal parameters were stored at \(-20^\circ C\) until assay.

For uniformity, results are expressed as mean \( \pm \) SEM. Two-sided \( t \)-tests (paired or unpaired, as appropriate) were used for statistical comparisons between groups; per variable, only one comparison was performed; significance level was set at \( P < 0.05 \).

Results

Baseline
Table I shows that total leukocyte count was relatively high in girls with incipient PCOS and in adolescents or young women with non-obese PCOS. In this age range, the relatively elevated leukocyte count was attributable to the absolute neutrophil count, which was on average \(>1000\) cells/mm\(^3\) above the norm. Lymphocytes, monocytes, basophils and eosinophils did not contribute significantly to the rise in leukocyte count.

| Table II. Treatment effects on leukocyte count were assessed in five randomized studies conducted in different phases of polycystic ovarian syndrome development |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Studies                        | 1       | 2       | 3       | 4       | 5       |
| Untreated                      | a       | b       | b      | c       | c       |
| Met                            | a       | b       | b      | c       | c       |
| Flu–Met                        |         |         |        |         |        |
| OC                             |         |         |        |         |        |
| OC plus Flu–Met                |         |         |        |         |        |
| Flu (mg/day)                   | 850     | 1275    | 1275   | 1275   | 850    |
| Met (mg/day)                   |         |         |        |         |        |

*Compared groups in Study 1. Compared groups (b) in Studies 2 and 3 were pooled in Table III, as were groups (c) in Studies 4 and 5.

Met = metformin; Flu = flutamide; OC = oral contraception.

| Table III. Baseline values in neutrophil and lymphocyte counts, and changes (0–3 months) following randomized treatments in three phases of polycystic ovarian syndrome development (total \( n = 118 \)) |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Treatment                      | \( n \)   | Age (years) | BMI (kg/m\(^2\)) | Neutrophils \((\times 1000/mm^3)\) | Lymphocytes \((\times 1000/mm^3)\) |
|                                | Baseline | Change | Baseline | Change | Baseline | Change |
| Untreated                      | 12       | 12.4 (0.3) | 21.0 (0.6) | 4.0 (0.4) | 0.6 (0.2) | 2.8 (0.2) |–0.1 (0.2) |
| Met                            | 12       | 12.5 (0.2) | 21.0 (0.7) | 4.6 (0.4) | –1.1 (0.2) | 2.5 (0.1) | 0.1 (0.2) |
| Untreated                      | 27       | 15.1 (0.3) | 21.0 (0.4) | 4.2 (0.2) | 0.1 (0.2) | 2.6 (0.1) |–0.1 (0.1) |
| Flu–Met                        | 26       | 15.3 (0.3) | 21.9 (0.4) | 4.2 (0.2) |–0.5 (0.2) | 2.7 (0.1) | 0.1 (0.1) |
| OC                             | 21       | 18.4 (0.3) | 22.0 (0.5) | 3.9 (0.2) | 0.8 (0.2) | 2.3 (0.2) |–0.1 (0.2) |
| OC plus Flu–Met                | 20       | 18.2 (0.3) | 22.0 (0.5) | 4.1 (0.2) |–0.9 (0.4) | 2.6 (0.2) |–0.3 (0.2) |

| Values are mean (SEM). |
| \( ^a \) \( P < 0.02 \) versus untreated. |
| \( ^b \) \( P < 0.001 \) versus untreated or OC. |
| NS = non-significant versus untreated or OC. |

Met = metformin; Flu = flutamide; OC = oral contraceptive.
Treatment

Table III summarizes the 3 month changes in neutrophil and lymphocyte counts observed after initiating randomized treatments. In none of the treatment groups were significant changes noted in monocyte, basophil or eosinophil counts (data not shown).

Study 1: untreated versus metformin in early post-menarche

The neutrophil count in metformin-treated girls with incipient PCOS (mean age 12.5 years) dropped by \( > 1000 \text{ cells/mm}^3 \) within 3 months (\( P < 0.001 \) versus baseline; \( P < 0.001 \) versus untreated), whereas the normal lymphocyte count was not detectably affected by metformin therapy.

Studies 2 and 3: untreated versus flutamide–metformin in adolescence

The high neutrophil count in PCOS adolescents treated with flutamide–metformin (mean age 15.2 years) dropped by \( \sim 500 \text{ cells/mm}^3 \) within 3 months (\( P < 0.02 \) versus untreated girls), whereas the normal lymphocyte count was not detectably affected by this treatment.

Discussion

There are two major strategies in the therapy for non-obese PCOS: one uses an OC from one of four generations of estrogen-progestagens, which also induce a SHBG rise; the other uses insulin sensitization and/or androgen receptor blockade, for example, with generics as metformin and/or low-dose flutamide (Dunaif et al., 1996; Nestler and Jakubowicz, 1997; de Leo et al., 1998; Diamanti-Kandarakis et al., 1998, 2003; Ibañez et al., 2000a,b, 2001b, 2002a, 2003, 2004a,b; Moghetti et al., 2000; Elter et al., 2002; Ibañez and de Zegher, 2003, 2004a, b; Morin-Papunen et al., 2003b; Gambineri et al., 2004; Guido et al., 2004; Palep-Singh et al., 2004).

In girls with incipient PCOS and in adolescents and young women with PCOS, we studied the effects of some of these treatments on leukocyte count. The results indicate: (i) that a relatively high leukocyte count is already present in girls and adolescents with hyperinsulinaemic hyperandrogenism, and is at this young age essentially attributable to an augmented neutrophil count; (ii) that this relative hyperneutrophilia is attenuated by metformin or low-dose flutamide–metformin, and is further amplified by OC monotherapy; and (iii) that, if these treatments are combined, the normalizing effects of flutamide–metformin overcome the OC effects on neutrophil count. These results remain to be confirmed in larger studies, in other ethnic populations and in other age ranges; until our findings have been corroborated in breadth and over time.

Table IV. Polycystic ovarian syndrome (PCOS) is characterized by a pro-inflammatory and pro-adipose state

<table>
<thead>
<tr>
<th>PCOS state</th>
<th>Effect of OC monotherapy</th>
<th>Effect of OC plus Flu–Met</th>
</tr>
</thead>
<tbody>
<tr>
<td>High neutrophil</td>
<td>None or ↑</td>
<td>↓ toward normal</td>
</tr>
<tr>
<td>High interleukin-6</td>
<td>None or ↑</td>
<td>↓ toward normal</td>
</tr>
<tr>
<td>Low adiponectin</td>
<td>None or ↓</td>
<td>↑ toward normal</td>
</tr>
<tr>
<td>Total fat excess</td>
<td>↑</td>
<td>↑ toward normal</td>
</tr>
<tr>
<td>Abdominal fat excess</td>
<td>↑</td>
<td>↑ toward normal</td>
</tr>
<tr>
<td>Deficit of lean mass</td>
<td>↑</td>
<td>↑ toward normal</td>
</tr>
</tbody>
</table>

Treatment of PCOS with an oral contraceptive (OC) in monotherapy results in changes away from the norm.

The addition of low-dose flutamide–metformin (Flu–Met) to the OC reverses the course towards the norm.

Quantitative results are in Tables I and III, and in Ibañez and de Zegher 2003, 2004b; Ibañez et al., 2003, 2004a,b.
they may be viewed as being of chiefly pathophysiological interest, rather than of immediately therapeutic relevance.

The present findings align well with those of earlier studies that used CRP or IL-6 as markers of the chronic, low-grade inflammation in PCOS (Morin-Papunen et al., 2003a, Ibáñez and de Zegher 2003, 2004a, 2005; Ibáñez et al., 2002a, 2003, 2004b). Collectively, these studies disclose the anti-inflammatory and anti-adipose benefit of giving metformin to girls with incipient PCOS, and of adding low-dose flutamide—metformin to an OC in young women with PCOS (Table IV). The anti-inflammatory effects of metformin and flutamide may underpin, or ensue from, the widely normalizing effects that these compounds have on hyperinsulinaemic hyperandrogenism, dyslipidaemia, body adiposity, elevated CRP and hypo-adiponectinaemia in PCOS patients (Diamanti-Kandarakis et al., 1998, Diamanti-Kandarakis et al., 2003; Ibáñez et al., 2002a, 2003, 2004b; Morin-Papunen et al., 2003a; Ibáñez and de Zegher, 2003, 2004a, 2005). Recently, there has been a remarkable increment in the recognition of inter-linkages among low-grade inflammation, insulin resistance, fat excess and endothelial dysfunction in PCOS and other hyperinsulinaemic states (Libby, 2002; Fernández-Real and Ricart, 2003; Goldfine and Kahn, 2003; Weisberg et al., 2003; Xu et al., 2003; Dandona et al., 2004; Fonseca et al., 2004; Tarkun et al., 2004; Orio et al., 2005; Sjöholm and Nyström, 2005). Regardless of the precise mechanisms involved, our study strengthens the evidence that metformin and flutamide—metformin may be regarded as, respectively, prime and adjuvant therapies for girls and young women with incipient or overt PCOS.

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