Flutamide–metformin for post-menarcheal girls with preclinical ovarian androgen excess: evidence for differential response by androgen receptor genotype

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Abstract

Objective: Addition of androgen receptor (AR) blockade (flutamide) to insulin-sensitising therapy (metformin) may confer synergistic benefits in girls with hyperinsulinaemic androgen excess. We hypothesised that girls with shorter AR gene CAG repeat alleles, and thus greater receptor sensitivity, might benefit more from the addition of low-dose flutamide.

Design: Open randomised crossover study.

Methods: In this study, 32 post-menarcheal girls (mean age 12.1 years) with a history of low birth weight and precocious pubarche were subgrouped by CAG genotype (‘short’: CAG mean length ≤20, n=14; ‘long’: CAG > 20, n=18). Within each subgroup, girls were 1:1 randomised to metformin alone (850 mg/day) or in combination with flutamide (62.5 mg/day) for 12 months. To allow comparisons with no treatment, long-CAG girls randomised to flutamide–metformin, and short-CAG girls randomised to metformin alone were observed for 12 months before treatment. Body composition by absorptiometry, fasting lipid profiles and levels of insulin, glucose and androgens were measured during the first 12 months on each treatment.

Results: In all girls, 12 months flutamide–metformin lowered body fat and improved lipid profiles when compared with no treatment. Compared with metformin alone, flutamide–metformin achieved greater reductions in the percentage of body fat and abdominal fat mass in the short-CAG subgroup (P < 0.001 to P < 0.0001). In contrast, in the long-CAG subgroup, flutamide–metformin produced no further improvements when compared with metformin alone.

Conclusions: In young post-menarcheal girls with preclinical androgen excess, low-dose flutamide–metformin improved body composition and key endocrine–metabolic abnormalities. However, only those girls with genetic markers of greater AR sensitivity may benefit from the addition of flutamide above metformin alone.

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Background

In adolescent girls, androgen excess is often accompanied by hyperinsulinaemia. It is unclear which of the two is the primary etiological factor. Rather, they appear to act synergistically in the development of hyperinsulinaemic hyperandrogenic states and their complications, such as fat excess and cardiovascular disease risk. Genetic candidates for androgen excess therefore include genes associated with increased activity of insulin or androgens. One example is the repeat number polymorphism on exon 1 of the androgen receptor gene (AR) CAG, which is functionally associated with AR sensitivity. Shorter AR CAG alleles confer increased AR sensitivity to ligand binding and have been associated with increased risks for conditions of androgen excess (1–5).

Consistent with the dual pathogenesis model involving both hyperandrogenism and hyperinsulinaemia, we first reported additive benefits of AR blockade with flutamide (250 mg/day) and insulin sensitisation with metformin (1275 mg/day) in non-obese, young women (mean age 19 years) with symptomatic hyperinsulinaemic androgen excess (6). Subsequently, in late-adolescent girls (mean age 16 years) with symptomatic hyperinsulinaemic hyperandrogenism, lower dose combinations of flutamide (62.5–125 mg/day) and metformin (850–1275 mg/day) were shown to decrease serum androgen levels, improve insulin sensitivity and lipid profiles, attenuate low-grade inflammation and decrease total body fat and abdominal fat mass within 3–9 months (7–13). Additive benefits of higher dose flutamide (500 mg/day) and metformin (1700 mg/day)
have also been described in obese women with symptomatic androgen excess (14, 15).

Our studies of girls with a history of both low birth weight (LBW) and precocious pubarche (PP; appearance of pubic hair before the age of 8 years) have described their high risks not only for early menarche (16), but also for post-menarcheal androgen excess and dyslipidaemia, associated with increased total and central body fat (17–20). Recognition of this clinical sequence led to previous trials of preventative interventions with metformin-alone post-menarche (21), or even pre-puberty (22, 23), to attenuate the characteristic development of endocrine–metabolic abnormalities, fat excess and clinical hyperandrogenism. However, the benefits of preventative flutamide therapy prior to the onset of clinical features of androgen excess have not yet been tested.

There is emerging evidence that low-dose flutamide (1–2 mg/kg per day) is not hepatotoxic in adolescents or women with androgen excess (24, 25). However, it remains desirable to restrict the use of flutamide to those individuals who are most likely to benefit. We hypothesised that any benefits of early AR blockade would be greater in girls with a genetically increased AR activity, namely those girls with shorter AR CAG repeat lengths.

We tested the dual hypothesis that in young post-menarcheal LBW–PP girls at risk for development of clinical hyperandrogenism: 1) low-dose flutamide–metformin safely reduces body adiposity and attenuates a spectrum of endocrine–metabolic abnormalities and 2) any additional benefits of low-dose flutamide (above metformin alone) are more readily conferred to girls with a more active AR variant, as indicated by a shorter AR CAG genotype.

### Subjects and methods

#### Study population and ethics

The study population consisted of 32 girls (age (mean ± S.E.M.): 12.1 ± 0.1 years; range 10.6–13.0 years). Clinical characteristics at baseline are summarised in Table 1.

The inclusion criteria were: 1) recently (6–12 months), post-menarcheal girls with a history of PP and LBW (birth weight for gestational age less than −1.5 S.D., or fifth percentile); this level of prenatal growth restraint in PP girls is associated with ovarian hyperandrogenism in adolescence (17); 2) body mass index < 26 kg/m² (26); 3) hyperinsulinaemia on a standard 2-h oral glucose tolerance test, defined as peak serum insulin level > 150 μU/ml, or mean serum insulin > 84 μU/l (27, 28); and 4) ovarian androgen excess, defined as a peak 17-OH-progesterone level > 160 ng/dl after gonadotrophin-releasing hormone agonist (leuprolide acetate; Procrin, Abbott, 500 μg s.c.) (29).

Mean age at PP diagnosis was 6.6 ± 0.1 years. PP was attributed to exaggerated adrenarche, based on high serum androstenedione and/or dehydroepiandrosterone sulphate (DHEAS) levels (17). Menarche had occurred 7.1 ± 0.1 months before study entry, and all girls had reached Tanner breast stage 4 or 5 (30).

### Table 1 Baseline endocrine–metabolic and body composition indices in early post-menarcheal girls (age ~ 12 years) with a history of low birth weight and precocious pubarche. Girls were classified by their mean androgen receptor gene CAG allele length: ≤ 20 (‘short CAG’) and > 20 (‘long CAG’).

<table>
<thead>
<tr>
<th>Reference</th>
<th>All girls (n=32)</th>
<th>Short CAG (n=14)</th>
<th>Long CAG (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight SDS</td>
<td>2.1 ± 0.1</td>
<td>2.1 ± 0.1</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Age at study start (years)</td>
<td>12.1 ± 0.1</td>
<td>11.9 ± 0.2</td>
<td>12.2 ± 0.2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.3 ± 0.3</td>
<td>21.1 ± 0.4</td>
<td>21.0 ± 0.6</td>
</tr>
<tr>
<td>Testosterone (ng/dl)</td>
<td>31 ± 3</td>
<td>74 ± 5</td>
<td>72 ± 5</td>
</tr>
<tr>
<td>SHBG (μg/dl)</td>
<td>1.9 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>DHEAS (μg/dl)</td>
<td>133 ± 15</td>
<td>143 ± 8</td>
<td>150 ± 13</td>
</tr>
<tr>
<td>Fasting insulin (μU/ml)</td>
<td>8.8 ± 0.4</td>
<td>11.6 ± 0.6</td>
<td>11.9 ± 0.9</td>
</tr>
<tr>
<td>Peak insulin on oGTT (μU/ml)</td>
<td>53.4 ± 3.0</td>
<td>100.1 ± 4.8</td>
<td>99.8 ± 8.3</td>
</tr>
<tr>
<td>MSII (ml/l)</td>
<td>45.7 ± 4.1</td>
<td>81.2 ± 3.4</td>
<td>81.1 ± 5.2</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dl)</td>
<td>70 ± 5</td>
<td>92 ± 3</td>
<td>90 ± 4</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dl)</td>
<td>62 ± 5</td>
<td>55 ± 2</td>
<td>57 ± 3</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>61 ± 4</td>
<td>72 ± 5</td>
<td>76 ± 6</td>
</tr>
<tr>
<td>IGFI (ng/ml)</td>
<td>80 ± 27</td>
<td>375 ± 10</td>
<td>371 ± 11</td>
</tr>
<tr>
<td>Percentage of fat mass</td>
<td>13.6 ± 1.4</td>
<td>35.4 ± 1.1</td>
<td>35.9 ± 1.8</td>
</tr>
<tr>
<td>Abdominal fat mass (kg)</td>
<td>2.5 ± 0.3</td>
<td>4.2 ± 0.3</td>
<td>4.6 ± 0.5</td>
</tr>
<tr>
<td>Lean mass (kg)</td>
<td>36.9 ± 1.3</td>
<td>32.6 ± 0.6</td>
<td>32.3 ± 0.8</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. There were no significant differences between the two CAG allele groups. BMI, body mass index; SHBG, sex hormone-binding globulin; DHEAS, dehydroepiandrosterone sulphate; oGTT, oral glucose tolerance test; MSII, mean serum insulin during an oGTT; IGF-I, insulin-like growth factor I.

*Normal references are taken from values in healthy volunteers matched for height and weight (n = 24 for endocrine–metabolic variables, age 15.3 ± 0.2 years; n = 10 for body composition, age 13.4 ± 0.1 years).

Low (≤ 20) or high (> 20) number of CAG repeats in exon 1 of the androgen receptor gene; short ≤ 20; long > 20 (Ref. (5)). To convert units to SI, multiply the concentrations of testosterone by 0.03467, those of androstenedione by 0.0349 and those of DHEAS by 0.02 714; divide the concentrations of SHBG by 0.0288, those of triglycerides by 89.5 and those of HDL cholesterol and LDL cholesterol by 38.7.
None of the girls had a family or personal history of diabetes mellitus or presented evidence for thyroid dysfunction. Cushing syndrome, hyperprolactinaemia, glucose intolerance (31), late-onset congenital adrenal hyperplasia (32, 33) or clinical signs or symptoms of androgen excess (34, 35); none was receiving an oestrogen-progesterone contraceptive, or any medication known to affect gonadal function or carbohydrate metabolism.

This study started in 2003, before prospective registration of randomised controlled trials, and thus no International Standard Randomized Controlled Trial Number was required. The study protocol was approved by the Institutional Review Board of Barcelona University, Hospital of Sant Joan de Déu. Informed consent was obtained from parents and assent from girls.

**Study design**

The design of this randomised open-label study is summarised in Fig. 1. Girls were subgrouped by their genotype at the polyglutamine (CAG) repeat polymorphism on exon 1 of AR. DNA extraction and genotyping have been described (5). Based on the previously used (5) CAG length cut-off, 14 girls had a mean CAG repeat length ≤20 (‘short-CAG’), and 18 girls had a mean CAG repeat length >20 (‘long-CAG’; Table 1). Mean CAG allele length ranged from 17 to 20 in the short-CAG group (mean ± s.e.m., 18.8 ± 0.3), and from 21 to 24 in the long-CAG group (mean ± s.e.m., 22.4 ± 0.2). Randomisation was performed with the Gran Mos program of the Institut Municipal d’Investigació Médica, Barcelona (22).

Girls in the short-CAG subgroup were 1:1 randomised to remain untreated (n=7) or to receive low-dose flutamide–metformin (flutamide 62.5 and metformin 850 mg once daily at dinner time; n=7) for 12 months. In the following 12 months, the initially untreated subgroup received only metformin (850 mg/d). The other subgroup remained on flutamide–metformin for a further 12 months, but only data from the first 12 month period were analysed.

Girls in the long-CAG subgroup were 1:1 randomised to remain untreated (n=9) or to receive metformin (850 mg/day; n=9) for 12 months. In the following 12 months, the initially untreated subgroup received flutamide–metformin (62.5 and 850 mg/day), and the other subgroup remained on metformin alone for a further 12 months, but only data from the first 12 month period were analysed.

This study design maximised the power of these 32 girls with available genotype data, as it allowed overall comparisons of the first 12 months only on each treatment between 16 untreated girls (comprising 7 short-CAG + 9 long-CAG both in year 1 of the study), 16 flutamide–metformin-treated girls (7 short-CAG in year 1 + 9 long-CAG in year 2) and 16 metformin-alone-treated girls (7 short-CAG in year 2 + 9 long-CAG in year 1). Each comparison therefore comprised a combination of paired and unpaired data, and we followed the standard statistical approach of treating all such data as unpaired (36).

**Clinical assessments and body composition**

Clinical examination was performed 6-monthly, together with assessment of body composition, fasting blood glucose and serum insulin, sex hormone-binding globulin (SHBG), DHEAS, androstenedione, testosterone, free androgen index (testosterone ×100/SHBG, an estimate of free testosterone), insulin-like growth factor-I (IGF-I) and lipid profile.

Body composition was assessed by dual-energy X-ray absorptiometry with a Lunar Prodigy (Lunar Corp., Madison, WI, USA). Absolute whole-body fat and lean mass were assessed (in kg), as well as fat content in the abdominal region, which was defined as the area between the dome of the diaphragm (cephalad limit) and the top of the greater trocanter (caudal limit) (37). Radiation dose per examination was 0.1 mSievert. Coefficients of variation (CV values) for scanning precision are estimated to be 2.0 and 2.6% for fat and lean body mass (Hologic, Waltham, MA, USA) with an intra-individual CV for abdominal fat mass of 0.7%. Indicative values of body composition were obtained from healthy Catalan schoolgirls, living in the same area and matched for age, body size and pubertal stage.

**Hormone assays**

Serum glucose was measured by the glucose oxidase method. Serum total cholesterol, high density lipoprotein (HDL) cholesterol and triglycerides were measured by the CHOD-PAP- and GPO-PAP-based methods, as described (5); low density lipoprotein (LDL) cholesterol was calculated by Friedewald formula. Serum insulin, DHEAS, androstenedione, testosterone, SHBG and IGF-I were assayed as described (22). All methods had intra- and inter-assay CV values between 4 and 8% within the
relevant concentration ranges. Samples were kept frozen at \(-20^\circ\text{C}\) until assay.

**Calculations and statistics**

Data on birth weight and gestational age were obtained from hospital records and transformed into SDS (17). In the Figures, for ease of display and to allow comparison of effect sizes in the various outcomes, changes in each of the body composition and endocrine–metabolic outcomes were expressed as z-scores, calculated by dividing the absolute changes by the corresponding baseline s.d. in the whole study population. Statistical analyses were based on raw values, unless indicated otherwise.

Two-sided t-tests were performed to compare baseline variables between CAG genotype subgroups, and also between treatment types. Changes in body composition and endocrine–metabolic features were compared over the first 12 months on each treatment.

Differential responses to metformin or flutamide–metformin therapy were compared between groups with different CAG length by testing the interaction between treatment type (metformin or flutamide–metformin) and CAG subgroup (long or short) on changes in outcome variables. There were no significant period effects, i.e. overall mean changes in the first 12 months were similar to the second 12-month study period. However, these could be underestimated due to low power. Therefore, to adjust for potential study period effects, SDS were recalculated using the overall means and s.d. at each relevant time point, i.e. at baseline, 12 months or 24 months, and analyses were repeated based on the changes in these SDS. The level of statistical significance was set at \(P<0.05\).

**Results**

At baseline, the 32 non-obese girls with an LBW–PP history and with current hyperinsulinaemia and androgen excess showed the expected abnormalities in body composition and endocrine–metabolic status (Table 1). However, these baseline variables did not differ between CAG length subgroups (Table 1). Mean biochemical and body composition variables for each subgroup at 0, 12 and 24 months are shown in Table 2. As described earlier, the following statistical comparisons were made between specific combinations of those subgroups over the first 12 months on each treatment.

**Flutamide–metformin versus no treatment**

Low-dose flutamide–metformin therapy \((n=16)\) for 12 months was associated with significant improvements in body composition, androgen levels and lipid profiles, compared with both baseline values and untreated girls.
(n=16) who continued to show worsening of these outcomes (comparisons are shown in Fig. 2).

Flutamide–metformin versus metformin alone in CAG genotype subgroups

In the short-CAG subgroup, flutamide–metformin resulted in more reduction in adiposity than metformin alone (percentage of total body fat: \( P = 0.005 \); percentage of abdominal fat: \( P = 0.005 \); Fig. 3, upper panel); no additional benefits of flutamide–metformin reached significance. In the long-CAG subgroup, after 12 months all improvements were comparable between flutamide–metformin and metformin alone (Fig. 3, lower panel).

When assessed in a combined multivariate model, there were significant interactions between treatment type (metformin or flutamide–metformin) and CAG subgroup (long or short) on changes in the percentage of total body fat (\( P = 0.004 \)) and abdominal fat (\( P = 0.0004 \)), indicating different treatment responses by genotype (Table 3). Repeat analyses using changes in period-specific SDS to allow for potential study period effects confirmed the significant treatment response interactions with CAG genotype for the percentage of total body fat (\( P = 0.001 \)) and abdominal fat mass (\( P < 0.001 \)).

Both flutamide–metformin and metformin-alone therapies were well tolerated; no significant side effects leading to medication or trial discontinuation occurred during the 24-month study. There were no changes in serum aspartate aminotransferase or alanine aminotransferase levels after 12 months of flutamide–metformin or metformin-alone therapies (Table 2), nor at the 6-month interim measurements (data not shown).

Discussion

In a randomised open-label intervention study, we observed that low-dose flutamide–metformin in young post-menarcheal LBW–PP girls (aged ~12 years) with hyperinsulinaemic androgen excess leads to striking improvements in body composition and endocrine-metabolic indices within 12 months. Compared with metformin alone, the addition of flutamide conferred further body composition benefits in the subgroup with higher AR activity, as indicated by shorter mean AR CAG allele lengths, but not in the other subgroup.

Decreasing AR CAG repeat number has been shown to increase transcriptional response to androgens in vitro (1, 38). In rat cell lines, complete removal of this tract resulted in a threefold increase in receptor transactivation, and there is a linear decline in receptor function with increasing CAG repeat length (38). In normal humans, AR CAG repeat number ranges between 11 and 35. Within the normal range, shorter CAG repeat number encoding increased androgen activity has been associated with increased prostate size and cancer risk in men (39, 40), and with variable risks for polycystic ovary syndrome (3, 4, 41) and breast cancer in women (42). Some studies have reported associations with preferential expression of longer CAG repeat alleles due to differential methylation; however, we did not assess methylation status in our study.

We previously described the clinical relevance of this functional polymorphism in 181 Barcelona girls who presented with PP, a condition associated with increased risk for hyperinsulinaemic androgen excess from adolescence onwards (35). In that study, PP girls had shorter mean CAG repeat number than 124 Barcelona control girls, and a greater proportion of short alleles...
differences seen in older girls (5) may appear with the increasing development of ovarian hyperandrogenism in untreated girls.

The present data, showing diverse benefits of flutamide–metformin on body composition and endocrine–metabolic variables in young post-menarcheal LBW–PP girls, are consistent with our previous results in older adolescents and women with hyperinsulinaemic androgen excess (6–13). Furthermore, our findings are consistent with the biologically plausible hypothesis that the additional benefits of AR blockade would be seen in the girls with higher genetic AR activity.

We do not claim that this small short-term trial represents a full pharmacogenetic study, as we did not assess the longer-term risk–benefit ratio of the flutamide–metformin combination in either subgroup. However, our findings suggest that a genetic marker for lower AR activity (i.e. longer CAG alleles) might help to exclude those individuals who are less likely to benefit from the addition of flutamide above metformin alone. Such exclusion may be useful in view of expressed concerns regarding the rare possibility of flutamide hepatotoxicity (43, 44), although there is at present no evidence of such side effects at low doses 1–2 mg/kg per day (24, 25).

The present findings strengthen the concept that, in adolescents and young women, excessive androgen action contributes to the accumulation of central fat (7–11, 45). Indeed, in the direct comparisons between flutamide–metformin and metformin alone, additional benefits of flutamide were seen on both the percentage of total body fat and abdominal fat mass. In a previous study in young women (age 18–22 years) with androgen excess, flutamide conferred additive benefits in reducing serum androgen levels when compared with metformin alone (6). In the current study of younger girls soon after menarche, it is possible that lower absolute levels and larger puberty-related fluctuations in androgen levels could have masked any direct reduction due to flutamide; body composition may be a cumulative and thus more sensitive marker of hormonal changes. Alternatively, the effects of flutamide on body

Table 3 Multiple regression models showing different responses in body composition according to both treatment type and androgen receptor CAG repeat genotype.

<table>
<thead>
<tr>
<th>B</th>
<th>S.E.M.</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Dependent variable: 12 months change in the percentage of total body fat</td>
<td>Intercept</td>
<td>0.729</td>
</tr>
<tr>
<td>Flu–Met (versus Met)</td>
<td>−3.439</td>
<td>1.063</td>
</tr>
<tr>
<td>CAG long (versus short)</td>
<td>−3.340</td>
<td>1.002</td>
</tr>
<tr>
<td>Flu–Met * short CAG</td>
<td>−4.383</td>
<td>1.418</td>
</tr>
<tr>
<td>(2) Dependent variable: 12 months change in abdominal fat mass (kg)</td>
<td>Intercept</td>
<td>0.538</td>
</tr>
<tr>
<td>Flu–Met (versus Met)</td>
<td>−1.002</td>
<td>0.185</td>
</tr>
<tr>
<td>CAG long (versus short)</td>
<td>−0.740</td>
<td>0.174</td>
</tr>
<tr>
<td>Flu–Met * short CAG</td>
<td>−1.194</td>
<td>0.246</td>
</tr>
</tbody>
</table>

B, regression coefficient. The significant interaction term (Flu–Met * short CAG) indicates a greater additional benefit of Flu–Met above metformin alone within the short CAG subgroup when compared with the long CAG subgroup.
fat and fat distribution could occur locally at the tissue level, without changes in circulating androgen levels. The study population (n = 32) was limited mainly due to the stringent inclusion criteria, and this may have limited the power to detect treatment type and genotype subgroup differences in the metabolic outcomes. However, the differential effects of flutamide–metformin on body composition by genotype were highly significant. In order to maximise the efficiency to test the multiple study comparisons (flutamide–metformin versus no treatment; flutamide–metformin versus metformin alone; and short-CAG versus long-CAG), the subgroups were randomised to different sequences of treatment type and initial observation period. Treatment effects were therefore often compared between different time periods. However, as the overall changes in all body composition and endocrine metabolic variables did not differ between time periods (i.e. changes in the whole group between 0 and 12 months were not different from changes between 12 and 24 months), this is unlikely to have caused any bias. Finally, the findings were confirmed on repeat analyses that adjusted for potential study period effects.

In conclusion, the efficacy and safety of low-dose flutamide–metformin therapy in conditions of hyperinsulinemic androgen excess are herewith extended to include young post-menarche girls aged ~12 years, before the development of overt clinical hyperandrogenism. Genetic markers of AR activity, such as AR CAG repeat alleles, contribute to identify those girls who are most likely to benefit from the addition of flutamide above metformin alone.

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