Association of aromatase (CYP 19) gene variation with features of hyperandrogenism in two populations of young women

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BACKGROUND: Aromatase catalyses the conversion of androgens to estrogens and thus variation in the aromatase gene could contribute to female syndromes of androgen excess, such as precocious pubarche (PP) and polycystic ovarian syndrome (PCOS).

METHODS: Two groups, one case–control containing girls from Barcelona, Spain with PP (n = 186) or healthy controls (n = 71), and the other a population study of young women from Oxford, UK, who volunteered for a study of normal women’s health (n = 109), were genotyped at four aromatase gene haplotype-tag single nucleotide polymorphisms (SNP). Clinical features and hormone concentrations relevant to hyperandrogenism were compared across haplotypes or genotypes.

RESULTS: Distributions of aromatase haplotypes (P < 0.0001) and aromatase SNP_50 genotype (P = 0.001) were significantly different between PP girls and Spanish controls. The AGGG haplotype was associated with an odds ratio (95% confidence interval) of 0.5 (0.3–0.9) (P = 0.005) for the presence of PP compared to GAGG. In 84 post-pubertal PP girls, aromatase haplotype was associated with functional ovarian hyperandrogenism (P < 0.05), independently of insulin sensitivity. In the Oxford population, SNP_50 was associated with variation in PCOS symptom score (P = 0.008) and circulating testosterone concentrations (P = 0.02).

CONCLUSIONS: This study suggests that common variation at the aromatase gene (and not just rare loss-of-function mutations) is associated with androgen excess in girls and young women.

Key words: genetic association/insulin resistance/polycystic ovarian syndrome/premature pubarche/testosterone

Introduction

Polycystic ovarian syndrome (PCOS) is characterized by hyperandrogenism and chronic anovulation, commonly leading to infertility. In some populations, women who develop clinical signs of ovarian androgen excess post-menarche present earlier with exaggerated adrenarche or precocious pubarche (PP), i.e. the appearance of pubic hair before the age of 8 years (Ibáñez et al., 1993, 2000). As with PCOS, girls with PP become insulin resistant and dyslipidaemic, particularly if PP was preceded by low birthweight (Ibáñez et al., 1998), suggesting that PP may be an antecedent of PCOS. Sisters of women with PCOS have increased circulating testosterone concentrations (Legro et al., 1998) and PP has been associated with genetic variation at the androgen receptor (Ibáñez et al., 2003), suggesting that hyperandrogenism in both PP and PCOS may be partly genetically determined.

Aromatase (EC 1.14.14.1) is a member of the cytochrome P450 family of enzymes (subfamily 19) which catalyses the conversion of C19 androgens to aromatic C18 estrogens. It is induced by FSH and is present in a number of different tissues including adrenals, muscle, placenta, skin, adipose and nervous tissue. Reduced aromatase activity may lead to the development of PCOS, since PCOS has been observed in patients with aromatase deficiency caused by rare loss-of-function mutations (Harada et al., 1992; Ito et al., 1993; Belgorosky et al., 2003) and antral follicles taken from PCOS women exhibited no aromatase activity (Takayama et al., 1996).

The human aromatase gene is ~130 kb long. Its 10 exons (the final nine of which are coding) are located within 30 kb of each other (Bulun et al., 2003), and the 93 kb 5′-flanking region is thought to have a regulatory role. In this study we explored the association between common variation in...
aromatase and hyperandrogenism in two separate populations of girls and young women, using a haplotype-tag single nucleotide polymorphism (htSNP) genotyping approach.

Materials and methods

Barcelona case–control study

All girls with PP were recruited from all cases that presented with a PP-like phenotype over a 5 year period. The policy at the Hospital Sant Joan de Déu (Barcelona, Spain) is to follow such patients auxologically until after completion of puberty, and to perform a hormonal assessment sometime pre-pubertally, in mid-puberty and after menarche. Girls were considered eligible for the study if they had PP due to premature adrenarche, as suggested by elevated plasma dehydroepiandrosterone sulphate (DHEAS) and/or androstenedione concentrations at clinical diagnosis (Ibañez et al., 1993). Enrolment exclusion factors included the presence of acanthosis nigricans, thyroid dysfunction, Cushing’s syndrome, androstenedione 6 and 11.9%, DHEAS 5.3 and 3.9%, SHBG 3.0% and 4.4%) were measured by radioimmunoassay or enzyme-linked immunosorbent assay (ELISA) kits as previously described (Ibañez et al., 1993, 1998, 2000). Glucose was measured using a glucose oxidase method. For the Oxford population study, plasma hormone concentrations (including testosterone inter-assay CV <10%, estradiol inter-assay CV <3%, androstenedione inter-assay CV <7%, 17-hydroxyprogesterone inter-assay CV <4%, SHBG inter-assay CV <5%, LH inter-assay CV <2%, FSH inter-assay CV <3%, insulin CV: intra-assay 8.3% and inter-assay 12.2%, and c-peptide intra-assay CV 3.4% and inter-assay CV 10.0%) were measured by radioimmunoassay or ELISA as previously described (Michelmore et al., 1999, 2001).

Aromatase genotyping

Linkage disequilibrium (LD) maps of aromatase single nucleotide polymorphisms (SNP) have been constructed to define htSNP that allow high predictability of haplotypes (Haiman et al., 2003). Four blocks of strong LD were found, the largest of which covered a 50 kb region containing the entire aromatase coding region and part of the 3’-untranslated region. The haplotypes in this region could be inferred from eight htSNP genotypes (Haiman et al., 2003). Close inspection, however, revealed that the increased diversity of aromatase in African-Americans alone accounts for four of the htSNP genotypes necessary to define this region (Haiman et al., 2003). In races such as Caucasians and Latinos, haplotypes in this LD-block could be inferred from just four htSNP genotypes, so we adopted this strategy for constructing aromatase coding-region haplotypes in our populations. For convenience, SNP genotypes are reported according to the nomenclature of Haiman et al. (2003), and genotyping was performed on genomic DNA that had been extracted from leukocytes (Ibañez et al., 1993; Michelmore et al., 2001). Replicate genotyping on the same DNA gave <1% inconsistent calls throughout.

SNP44 genotyping

SNP 44 (Haiman et al., 2003) (hCV8234947, rs12907866) was genotyped using PCR amplification and detection of SNP alleles by restriction fragment length polymorphism (RFLP) analyses. DNA (10ng) was amplified (20 μl total volume) along with 1 × reaction buffer, 200 μmol/l each dNTP (Promega Ltd, UK), 1 mmol/l magnesium, 12 pmol each primer (forward: 5’-GGTACCAGGTAGAGAAATGAGAAAC-3’; reverse: 5’-CCCTGTCATGGTCTTCTC-
AAGACAGATATA-3'), 10% (v/v) glycerol and 0.5 IU Biotaq DNA Polymerase (Bioline, UK). The mix was heated to 94°C for 5 min, followed by 20 cycles of 94°C (45 s), 59°C (45 s, dropping 0.5°C per cycle) and 72°C (45 s). After this the samples underwent 15 cycles of 94°C (45 s), 49°C (45 s) and 72°C (45 s), prior to a final incubation at 72°C for 10 min. Ten microlitres of the resulting PCR product was incubated at 65°C for 16 h along with 5 IU of Taq (New England Biolabs, UK). Separating the resulting products by agarose gel electrophoresis produced a 425 bp band for the ‘A’ allele and 258 and 167 bp bands for the ‘G’ allele.

**SNP50 genotyping**

SNP 50 (Haiman et al., 2003) (rs2414096) was genotyped using mismatch primer PCR–RFLP (Hao et al., 2002). DNA (10 ng) was amplified (20 μl total volume) along with 1 × reaction buffer, 200 μmol/l each dNTP, 1.5 mmol/l magnesium, 12 pmol of each primer (forward: 5′-TTGTATCCCCAAGAAAGCACC-3′; reverse: 5′-CTCAAACTCAATCTAGAGGCTCAAAG-3′), 5% (v/v) dimethylsulphoxide and 0.5 IU Biotaq DNA Polymerase. The mix was heated to 94°C for 5 min, followed by 20 cycles of 94°C (45 s), 55°C (45 s, dropping 0.5°C per cycle) and 72°C (20 s). After this the samples underwent 15 cycles of 94°C (45 s), 45°C (45 s) and 72°C (20 s), prior to a final incubation at 72°C for 10 min. Of the resulting PCR product, 10 μl was incubated at 60°C for 16 h along with 2.5 IU of BsiEI (New England Biolabs). This produced a 124 bp band for the ‘A’ allele and 102 and 22 bp bands for the ‘G’ allele.

**SNP60 genotyping**

SNP 60 (Siegelmann-Danieli and Buetow, 1999; Haiman et al., 2003) (int 7.14A, IVS7 200A) was genotyped using tetramer primer amplification refractory mutation system (ARMS) PCR (Ye et al., 2003). DNA (30 ng) was amplified (10 μl total volume) along with 1 × reaction buffer, 200 μmol/l each dNTP, 2.5 mmol/l magnesium, 10 pmol each inner primer (forward: 5′-CACAGTCAATACATATGTCATTGA-3′; reverse: 5′-AGGGACTGACCTGAACTAAGTC-3′) and 1 pmol each outer primer (forward: 5′-TGTTACTGACAGAAGAAGTATG-3′; reverse: 5′-TAGACAAATTGCTATTGGACACAGCCG3′-3′), 10% (v/v) glycerol and 0.5 IU Biotaq DNA Polymerase. The mix was heated to 94°C for 5 min, followed by 20 cycles of 94°C (45 s), 57°C (45 s, dropping 0.5°C per cycle) and 72°C (45 s). After this the samples underwent 15 cycles of 94°C (45 s), 47°C (45 s) and 72°C (45 s), prior to a final incubation at 72°C for 10 min. Of the resulting PCR product, 10 μl was incubated at 60°C for 16 h along with 2.5 IU of BsiEI (New England Biolabs). This produced a 124 bp band for the ‘A’ allele and 102 and 22 bp bands for the ‘G’ allele.

**SNP64 genotyping**

SNP 64 (Haiman et al., 2003) (rs4646) was also genotyped by ARMS PCR. The cycling conditions were similar to those for SNP 60, except that the annealing temperatures were 7°C higher throughout. The reaction mix was the same as for SNP 60 with the exception of the inner primer sequences (forward: 5′-GGTTGTTCACCCAAAGCTAGTGCTACTT-3′; reverse: 5′-TTTGGACAGGAGCAGATGGCC-3′) and outer primer sequences (forward: 5′-ACCCCAAGAACTCAGACAGGTGTCTG-3′; reverse: 5′-TGTTTAATGAGGGCCTATCC-3′). This produced a 223 bp band for the ‘G’ allele and a 176 bp band for the ‘T’ allele (with a full PCR product band at 346 bp).

### Statistical analyses

All genotype frequencies were assessed for consistency with Hardy–Weinberg equilibrium (the Barcelona samples being assessed separately for cases and controls due to the possibility of natural selection occurring for these loci in the cases). Statistical analyses were performed using SPSS for windows (version 10.0.7) (SPSS Inc., USA). In the Barcelona study there was 80% statistical power to be able to detect a difference of 0.4 SD of a quantitative variable at the quoted SNP 50 allele frequencies. For the Barcelona SNP genotypes there was 80% statistical power to detect a difference of 0.6 SD of a quantitative variable at the quoted SNP 50 allele frequencies. Aromatase coding-region haplotypes were reconstructed from the four SNP genotypes using Phase (version II) software (Stephens et al., 2001). For both studies, haplotypes were reconstructed using data from the complete set of samples (i.e. in Barcelona both the cases and controls combined and in the Oxford population the full group including those taking oral contraceptives). Haplotypes were expressed in terms of aromatase alleles in the order SNP 44, 50, 60 then 64. Hormone concentrations and quantitative phenotypic data were compared across aromatase genotypes and haplotypes by one-way ANOVA. Where appropriate, adjustment was made for insulin sensitivity [calculated as homeostatic model assessment (HOMA) (Levy et al., 1998) from fasting plasma insulin and glucose concentrations], age (at presentation or assessment), puberty stage (when analysing puberty-related hormone concentrations), body mass index (BMI) or birthweight. In the Barcelona study, frequencies of aromatase haplotypes were compared using the χ² goodness-of-fit test. Frequencies of individual haplotypes were compared (presence against absence in cases and controls) by the χ²-test.

### Results

**Barcelona case–control study**

Table I shows the baseline phenotypic and biochemical characteristics of the PP girls and controls. Table II shows the frequencies of their aromatase genotypes and haplotypes (along with those from the Oxford population of young women). The distributions of haplotypes (P < 0.0001) and SNP 50 genotypes (P = 0.001) were significantly different between PP girls and controls. The odds ratios with 95% confidence intervals are shown in Table II.

#### Table I. Phenotypic and biochemical characteristics of precocious pubarche (PP) girls and controls from Barcelona

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 71)</th>
<th>Girls with PP (n = 186)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at assessment (years)</td>
<td>11.2 (9.5–13.2)</td>
<td>10.3 (9.8–10.9)</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.42 (1.12–1.73)</td>
<td>1.43 (1.11–1.75)</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>Body mass index (SD score)</td>
<td>0.34 (0.05–0.72)</td>
<td>1.03 (0.81–1.25)</td>
<td>0.002</td>
</tr>
<tr>
<td>Serum testosterone (nmol/l)</td>
<td>1.0 (0.9–1.2)</td>
<td>1.4 (1.3–1.5)</td>
<td>0.007</td>
</tr>
<tr>
<td>Serum DHEAS (μmol/l)</td>
<td>0.24 (0.21–0.36)</td>
<td>0.29 (0.28–0.31)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Serum androstenedione (nmol/l)</td>
<td>2.96 (2.36–3.70)</td>
<td>4.45 (3.83–5.19)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Data are geometric mean (95% confidence interval). DHEAS = dehydroepiandrosterone sulphate.
DHEAS = dehydroepiandrosterone sulphate; HOMA = homeostasis model assessment.

Frequencies of all the aromatase SNP genotypes in these samples were generally consistent with Hardy–Weinberg equilibrium. However, SNP 50 genotypes from girls with PP were not in Hardy–Weinberg equilibrium ($P < 0.005$), unlike genotypes from the Spanish controls which were. Variation in this SNP genotype was not associated with variation in estradiol, insulin sensitivity or birthweight, but SNP 50 genotype was associated with plasma testosterone concentrations, after adjustment for puberty stage ($P = 0.03$; Table III) and this persisted after adjustment for birthweight SDS ($P = 0.03$). Adjusting for puberty stage also revealed significant associations with DHEAS concentrations ($P = 0.018$) and insulin sensitivity ($P = 0.039$; Table III).

In 84 post-pubertal PP subjects for whom data were available, aromatase haplotype was related to functional ovarian hyperandrogenism ($P = 0.049$), after adjustment had been made for insulin sensitivity (with reduced insulin sensitivity itself being related to functional ovarian hyperandrogenism, $P = 0.027$).

None of the other genotypes and haplotypes were associated with clinical or biochemical features of hyperandrogenism in this group.

### Oxford population study

All aromatase SNP genotypes in this cross-sectional study were consistent with Hardy–Weinberg equilibrium. The SNP 50 genotype was associated with PCOS symptom scores ($P = 0.008$ with dominant model; Table IV), but the aromatase coding-region haplotype was not associated with any clinical or biochemical feature of hyperandrogenism (all $P > 0.05$; SNP genotype and haplotype prevalences are shown in Table II). This association with SNP 50 was independent of BMI, insulin sensitivity and ultrasound diagnosis of polycystic ovaries, all of which were independently associated with the PCOS symptom score (all $P < 0.03$). The SNP 50 genotype was also associated with variation in circulating testosterone concentrations ($P = 0.02$ with dominant model; Table IV). There was no detectable association between this genotype and other features of PCOS or with insulin sensitivity ($P = 0.7$), even after adjusting for BMI ($P = 0.6$). Variation in estradiol quartile was also associated with

### Table IIa. Frequencies of genotypes in aromatase single nucleotide polymorphisms (SNP) expressed in number (%) of subjects in precocious pubarche (PP) girls and controls from Barcelona and young women from Oxford

<table>
<thead>
<tr>
<th>Aromatase SNP</th>
<th>Genotype</th>
<th>Barcelona controls</th>
<th>Barcelona girls with PP</th>
<th>Oxford young women</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP 44</td>
<td>A/A</td>
<td>53 (35)</td>
<td>66 (35)</td>
<td>76 (34)</td>
</tr>
<tr>
<td></td>
<td>A/G</td>
<td>77 (51)</td>
<td>87 (47)</td>
<td>115 (52)</td>
</tr>
<tr>
<td></td>
<td>G/G</td>
<td>21 (14)</td>
<td>34 (18)</td>
<td>31 (14)</td>
</tr>
<tr>
<td>SNP 50*</td>
<td>A/A</td>
<td>39 (26)</td>
<td>82 (44)</td>
<td>41 (19)</td>
</tr>
<tr>
<td></td>
<td>A/G</td>
<td>73 (48)</td>
<td>59 (32)</td>
<td>125 (56)</td>
</tr>
<tr>
<td></td>
<td>G/G</td>
<td>39 (26)</td>
<td>46 (25)</td>
<td>56 (25)</td>
</tr>
<tr>
<td>SNP 60</td>
<td>A/A</td>
<td>2 (1)</td>
<td>5 (3)</td>
<td>3 (1)</td>
</tr>
<tr>
<td></td>
<td>A/G</td>
<td>24 (16)</td>
<td>23 (13)</td>
<td>42 (19)</td>
</tr>
<tr>
<td></td>
<td>G/G</td>
<td>125 (83)</td>
<td>156 (85)</td>
<td>177 (80)</td>
</tr>
<tr>
<td>SNP 64</td>
<td>G/G</td>
<td>82 (54)</td>
<td>111 (59)</td>
<td>120 (54)</td>
</tr>
<tr>
<td></td>
<td>G/T</td>
<td>58 (38)</td>
<td>62 (33)</td>
<td>89 (40)</td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>11 (7)</td>
<td>14 (7)</td>
<td>13 (6)</td>
</tr>
</tbody>
</table>

* $P = 0.001$, Barcelona controls versus girls with PP.

### Table IIb. Frequencies of aromatase haplotypes expressed as number (%) of chromosomes in precocious pubarche (PP) girls and controls from Barcelona and young women from Oxford

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Barcelona controls</th>
<th>Barcelona girls with PP</th>
<th>Oxford young women</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAGG**</td>
<td>26 (9)</td>
<td>58 (16)</td>
<td>31 (7)</td>
</tr>
<tr>
<td>AGGG*</td>
<td>78 (26)</td>
<td>69 (18)</td>
<td>116 (26)</td>
</tr>
<tr>
<td>AGGT</td>
<td>45 (15)</td>
<td>50 (13)</td>
<td>67 (15)</td>
</tr>
<tr>
<td>AGAT</td>
<td>26 (9)</td>
<td>26 (7)</td>
<td>44 (10)</td>
</tr>
<tr>
<td>GAGG</td>
<td>116 (39)</td>
<td>150 (40)</td>
<td>164 (37)</td>
</tr>
<tr>
<td>Other haplotypes</td>
<td>9 (3)</td>
<td>21 (6)</td>
<td>22 (5)</td>
</tr>
</tbody>
</table>

There was a significant difference in haplotype distribution between Barcelona PP girls and controls ($P < 0.0001$).

* $P < 0.05$, ** $P < 0.01$ haplotype frequency in girls with PP versus controls.
The SNP 50 genotype ($P = 0.014$ with additive model; Table IV) but it was not related to the estradiol to testosterone ratio ($P = 0.4$).

There were no associations between variation in these phenotypic variables and variation in aromatase SNP 44, 60 or 64 (Table IV).

Discussion

This study demonstrates that common genetic variation in the coding region of aromatase is associated with risk for prepubertal hyperandrogenism, and with PCOS risk in both girls and young women. More specifically in our groups an intronic SNP close to exon 3 in aromatase (SNP 50) was associated with PP, post-menarchal ovarian hyperandrogenism and variation in PCOS symptom score. These associations could be explained by the additional association with circulating testosterone concentrations in the two separate groups.

The aetiology of PP, ovarian hyperandrogenism and PCOS has been linked to insulin resistance and obesity (Dunaif, 1997). Our findings were independent of insulin sensitivity and BMI. Genotype distributions between PP girls and healthy controls were different, with the SNP 50 ‘A’ allele (which was more prevalent in PP girls) being associated with increased testosterone concentrations in both the Barcelona PP case–control study and the Oxford population study (where it was also associated with increased PCOS symptom scores). Aromatase coding-region haplotypes in both our Barcelona controls and Oxford young women showed similar prevalences to those of Haiman et al. (2003). Variation in them was also shown to be associated with alterations in risk for PP, itself a risk factor for PCOS (Ibáñez et al., 1993, 2000). In particular, comparing haplotypes differing in the SNP 50 genotype, one variant was associated with a 2-fold decreased risk for having PP. In 84 post-pubertal subjects, reduced insulin sensitivity and aromatase coding region haplotypes were independently associated with functional ovarian hyperandrogenism risk. The lack of confirmation of the haplotype effects in the Oxford population study may well have been due to a lack of sufficient statistical power, with the four SNP genotyped being resolved into nine different haplotypes in that group of only 109 young women.

The significant findings in this study appear to relate to the SNP 50 genotypes. The question therefore arises as to whether this SNPS is causing these associations directly or whether it is just in LD with another causal SNPS. Clearly there are many SNPS in this aromatase block that are in strong LD (Haiman et al., 2003), hence the relatively small number of SNP that need to be typed to be used as haplotype tags. Indeed one SNP, only 667 base pairs away from SNP 50 [SNP 51 in Haiman et al. (2003)] in exon 3 of the gene, is in strong LD with SNP 50 (Haiman et al., 2003) and has already been shown to be associated with breast cancer risk in women (Siegelmann-Danieli and Buetow, 1999) and with circulating estrogen concentrations (Somner et al., 2004).

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While our samples sizes may be relatively small for genetic association studies, the detailed phenotype assessments in both populations would increase the power to identify true positive associations. The consistent associations in two very separate populations further increases the validity of the findings and reduces the likelihood of population stratification bias, although family-based transmission studies would be needed to confirm this. The Barcelona control girls were not recruited from a completely unselected population; however, they had no evidence of endocrine or any other
pathology. Similarly, it is possible that the Oxford volunteers may have over-selected themselves for the presence of PCOS features, no selection bias was detected (Michelmore et al., 2001), and as in the Barcelona control girls, the genotype and haplotype frequencies were similar to those observed in other populations (Haiman et al., 2003). Our associations were largely with hyperandrogenism, and future case–control studies would be needed to confirm the relevance of these findings to actual PCOS risk. Such studies would need to be much larger, in view of the widely heterogeneous pathogenesis of PCOS.

Previously described rare loss-of-function mutations in aromatase have demonstrated that variation in this estrogen biosynthesis pathway can indeed lead to PCOS in females (Harada et al., 1992; Ito et al., 1993; Conte et al., 1994; Morishima et al., 1995; Mullis et al., 1997; Belgorosky et al., 2003). So far the aromatase mutations described have been in exons 5 (Belgorosky et al., 2003), 9 (Belgorosky et al., 2003; Morishima et al., 1995; Mullis et al., 1997) and 10 (Ito et al., 1993; Conte et al., 1994), and in the boundary between exons and introns 3 (Mullis et al., 1997) and 6 (Harada et al., 1992). Our findings which relate to SNP 50, which is also in the coding-region, suggest that more subtle, common variations in aromatase are also associated with variation in risk for PCOS. Overall PCOS risk may involve oligogenic contributions (Franks et al., 2001) [as well as environmental components (Crosignani and Nicolosi, 2001)] including that made by aromatase, in addition to other genes such as those of the androgen receptor (Ibáñez et al., 2003), insulin receptor (Tucci et al., 2001), cholesterol side-chain cleavage enzyme (Gharani et al., 1997), follistatin (Urbanek et al., 1999) and the insulin VNTR (Michelmore et al., 2001). Alternatively, as PCOS is such a heterogeneous disorder, different genetic variants could associate with PCOS risk in different populations (Sanders et al., 2002).

In summary, this is the first association study that has found a link between genetic variation in the aromatase gene and androgen excess in females. This was confirmed in two independent populations, one a population survey and the other a case–control study. The fact that these two study groups, which gave very similar associations with aromatase genotypes, were established by different selection criteria gives these findings extra credence. Previously both a linkage (Gharani et al., 1997) and an association (Urbanek et al., 1999) study failed to find a relationship between aromatase and PCOS, but these studies were performed without the power associated with the use of hiSNPs and therefore some of the haplotypic variation in the gene may have been missed.

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References


Ibáñez L, Ong KK, Mongan N, Jaakelainen J, Marcos MV, Hughes IA, De Zegher F and Dunger DB (2003) Androgen receptor gene CAG repeat polymorphism in the development of ovarian hyperandrogenism. J Clin Endocrinol Metab 88,3333–3338.


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