COMMENT

The Methionine 196 Arginine Polymorphism in Exon 6 of the TNF Receptor 2 Gene (TNFRSF1B) Is Associated with the Polycystic Ovary Syndrome and Hyperandrogenism

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Inflammatory cytokines such as TNFα may play a role in the pathogenesis of common metabolic disorders, including hyperandrogenism and the polycystic ovary syndrome (PCOS). The TNF receptor 2 mediates most of the metabolic effects of TNFα. In the present study, we have evaluated serum soluble TNF receptor 2 levels, and several common polymorphisms in the TNF receptor 2 gene (TNFRSF1B), in women presenting with PCOS or hyperandrogenic disorders. Initial studies included 103 hyperandrogenic patients (42 presenting with PCOS) and 36 controls from Spain. The 196R alleles of the M196R (676 T → G) variant in exon 6 of TNFRSF1B, which is in linkage disequilibrium with a CA-repeat microsatellite polymorphism in intron 4 of TNFRSF1B, tended to be more frequent in hyperandrogenic patients than in controls (P = 0.056), reaching statistical significance when the analysis was restricted to include only PCOS patients (P < 0.02). Extended analysis including another 11 hyperandrogenic patients from Spain and 64 patients and 29 controls from Italy confirmed the association between 196R alleles of the M196R variant and hyperandrogenic disorders (P < 0.05), which was maintained when restricting the analysis to PCOS patients (P < 0.02). On the contrary, the 3′-untranslated region (exon 10) variants 1663 G → A, 1668 T → G, and 1690 T → C were not associated with hyperandrogenism. The soluble TNF receptor 2 levels were not different between patients and controls but were increased in obese subjects, compared with lean individuals, and were affected by the interaction between the 1663 G → A and 1668 T → G variants in the 3′-untranslated region of TNFRSF1B. The TNFRSF1B genotype did not influence any clinical or biochemical variable related to hyperandrogenism or insulin sensitivity and was not associated with obesity, both in hyperandrogenic patients and healthy controls considered separately. In conclusion, the M196R (676 T → G) variant in exon 6 of TNFRSF1B is associated with hyperandrogenism and PCOS, further suggesting a role for inflammatory cytokines in the pathogenesis of these disorders. (J Clin Endocrinol Metab 87: 3977–3983, 2002)

Inflammatory cytokines such as TNF-α play a role in the pathogenesis of metabolic disorders, such as obesity and insulin resistance-related disorders (1). TNF-α participates in obesity-related systemic insulin resistance by inhibiting the insulin receptor tyrosine kinase in muscle and fat, which are the two tissues mainly responsible for insulin-stimulated glucose uptake (2, 3). Hyperandrogenism and the polycystic ovary syndrome (PCOS) are common disorders in reproductive-aged women (4, 5) and are also included among the constellation of consequences of insulin resistance (6).

We have recently described that the TNF-α axis may also influence the pathogenesis of hyperandrogenism, independently from insulin resistance and obesity (7). Serum TNF-α levels were mildly increased in hyperandrogenic patients, especially in those with idiopathic hirsutism (7). Also, when considering patients and controls as a whole, carriers of the A allele of the common −308G → A single nucleotide polymorphism in the promoter of the TNF-α gene presented with increased serum androgen levels (7). However, −308A alleles were equally distributed among patients and controls, suggesting that this variant is only a contributing factor to the pathogenesis of androgen excess, instead of being a major etiology for this disorder (7).

TNFα actions are mainly mediated by type 1 (p55 or p60, TNFR1) and type 2 (p75 or p80, TNFR2) TNF receptors (TNFRs). The mRNAs of both receptors are up-regulated in adipose tissue of obese women, correlating with body mass index (BMI) and several indirect markers of insulin resistance (8, 9). Studies in genetically obese animals lacking these receptors have shown that TNFR1 is the major mediator of the TNFα induced insulin resistance and TNFR2 might potentiate this effect (10). On activation of the TNFα system soluble forms of TNFRs are shed into the circulation and may be measured by specific immunoas-

Abbreviations: A, Δ4-Androstenedione; BMI, body mass index; CI, confidence interval; DHEAS, dehydroepiandrosterone-sulfate; FIRI, fasting insulin resistance index; PCOS, polycystic ovary syndrome; sTNFR2, soluble TNF receptor 2; TNFR, TNF receptor; UTR, untranslated region.
says. The circulating levels of soluble TNFR2 (sTNFR2), but not those of soluble TNFR1, are increased in obese subjects, correlating with indexes of insulin resistance and serum lipid variables (11–14). However, most of these correlations seem to be derived from the stronger correlation with BMI because serum sTNFR2 levels are mainly determined by adiposity and genetic factors (15).

Variability at the gene encoding TNFR2 (TNFRSF1B, located at chromosome 1p36.2), influences sTNFR2 levels (16, 17) and has been proposed to play a role in the pathogenesis of several metabolic disorders, including familial combined hyperlipidemia (17), hypertension (16), obesity and insulin resistance (18), and coronary artery disease (19).

In the present study, we evaluated sTNFR2 levels and several common polymorphisms in TNFRSF1B in a large series of hyperandrogenic patients and healthy controls from Spain and Italy to further explore the role of the TNFα system in the pathogenesis of hyperandrogenism and PCOS.

Materials and Methods

Subjects

Initial studies included 103 hyperandrogenic patients from Spain recruited prospectively, who presented with hirsutism and/or hyperandrogenism. As stated above, because our previous finding of increased serum TNFα levels in hyperandrogenic women was not restricted to PCOS patients (7), we decided to study the possible role of sTNFR2 in women presenting with a larger spectrum of hyperandrogenic disorders, focusing secondarily on the subset of women presenting with PCOS. The control group of 36 nonhyperandrogenic women included lean female volunteers and patients attending the clinical practice of one of the authors (H.F.E.-M.) for the treatment of obesity. None of the controls had signs or symptoms of hyperandrogenism, menstrual dysfunction, or history of infertility.

Hirsutism, as defined by a modified Ferriman-Gallwey score (20) of 8 or more, was present in 98 of the patients, with a mean score of 15.2 ± (SD) 5.3. PCOS, defined by clinical and/or biochemical hyperandrogenism, oligomenorrhea, and exclusion of other etiologies (21), was present in 42 patients, including the 5 nonhirsute patients. Forty-four patients had hyperandrogenemic hirsutism, defined by hirsutism, increased serum androgen levels, and regular menses, and 17 patients presented with idiopathic hirsutism, defined by hirsutism, normal androgen levels, and regular menstrual cycles.

Because the initial study suggested an association of hyperandrogenism with one of the genomic variants in TNFRSF1B, the study was extended to increase the sample size and the statistical power with the inclusion of another 11 consecutive Spanish patients (eight presenting with PCOS, two with hyperandrogenemic hirsutism, and one with idiopathic hirsutism) as well as 64 hyperandrogenic patients recruited prospectively (58 presenting with PCOS, 2 with hyperandrogenemic hirsutism, and 4 with idiopathic hirsutism) and 29 healthy controls from northeastern Italy.

None of the patients had features of Cushing’s disease or drug-induced hirsutism. Hyperprolactinemia and congenital adrenal hyperplasia were ruled out because all the patients presented with basal serum PRL levels less than 24 µg/liter and ACTH-stimulated 17-hydroxyprogesterone levels less than 30 nmol/liter (22). All the controls and patients had blood pressure less than 140/90 mm Hg and fasting glucose levels less than 6.1 mmol/liter.

The patients and controls had not taken hormonal medications, including oral contraceptives, for the last 6 months. All the subjects were Caucasian. Data from some of the Spanish patients and controls, regarding different aspects of the pathophysiology of hirsutism, have been previously published (7, 23–27).

The ethics committees of the Hospital Ramón y Cajal and the University of Verona approved the study, and informed consent was obtained from each patient and control.

Study protocol and hormone profiles

Studies were performed during the midfollicular phase of the menstrual cycle or during amenorrhea, after excluding pregnancy by proper testing. In all the subjects, with the exception of 11 Italian controls, fasting basal serum samples were obtained for the measurement of total T, SHBG, Δ4-androstenedione (A), dehydroepiandrosterone-sulfate (DHEAS), glucose, and insulin. Samples were immediately centrifuged, and serum was separated and frozen at −20°C until assayed. The samples from the 103 patients and 36 controls from Spain initially studied were also assayed for serum sTNFR2.

Serum sTNFR2 levels were measured by ELISA kit (Quantikine human sTNF RII kit, R&D Systems, Minneapolis, MN) with a lower limit of detection of less than 1.0 pg/ml, and mean intra- and inter-assay coefficients of variation of 2.2% and 4.1%, respectively. Serum glucose was measured by the glucose oxidase method. Serum insulin, A, DHEAS, and SHBG were measured by commercial immunoassays (Diagnostic Products, Los Angeles, CA). Intra- and interassay coefficients of variation were 4.3% and 5.4% for insulin, 5.7% and 8.4% for A, 5.2% and 12.0% for DHEAS, and 6.5% and 8.7% for SHBG. Total T was assayed by direct RIA using commercial assays: Spectria Total Testosterone (Orion Diagnostica, Espoo, Finland) was used for Spanish samples, with intra- and inter-assay coefficients of variation of 5.3% and 5.4%, and Coat-A-Count Total Testosterone (Diagnostic Products) was used for Italian samples, with intra- and interassay coefficients of variation of 6.5% and 8.5%. The linear regression analysis of the two methods was established by measuring 12 samples, yielding the following statistics: Spectria T = Coat-A-Count T × 0.792 + 0.64 nmol/liter, r = 0.956, means 2.3 nmol/liter by Spectria, and 2.2 nmol/liter by Coat-A-Count.

The free T concentration was calculated from total T and SHBG concentrations, assuming a serum albumin concentration of 43 g/liter, and taking a value of 1 × 10⁻⁵ liters/mol for the association constant of SHBG for total T and a value of 3.6 × 10⁻⁵ liters/mol for that of albumin for total T (28). Insulin resistance in the fasting state was estimated from glucose and insulin levels using the fasting insulin resistance index (FIRI) [FIRI = glucose (millimoles/liter) × insulin (milliunits/liter)/25] (29).

DNA extraction and genotype analysis

Genomic DNA was extracted from leukocytes obtained from whole-blood samples, using commercial DNA purification kits (Wizard Genomic DNA purification kit, Promega Corp., Madison, WI, and Nucleon BAC C3, Amersham Pharmacia, Buckinghamshire, UK). Genomic variants in TNFRSF1B were numbered on the basis of their location in the TNFR2 mRNA sequence of GenBank accession number M23215. The 3' untranslated region (UTR) (exon 10) variants 1663 G→A, 1668 T→G, and 1690 T→C (30) were identified by direct sequencing after PCR amplification.

The CA repeat in intron 4 was identified by capillary electrophoresis of PCR products using fluorescence-labeled primers (17) using an ABI310 genetic analyzer (Applied Biosystems, Foster City, CA). Four alleles of 267, 269, 271, and 273 bp were identified and termed CA13, CA14, CA15, and CA16, reflecting the number of CA repeats (31).

The M196R (676 T→G) variant in exon 6 was detected by PCR-restriction fragments length polymorphism analysis (16) using NlaIII restriction enzyme. The G allele was not cut by NlaIII and appeared as a single band (344 bp) in agarose Tris-borate-EDTA gels, whereas the T allele was cut and appeared as two bands (235 and 109 bp).

Statistical analysis

Data are represented as mean ± SD unless otherwise stated. The Kolmogorov-Smirnov statistic, with a Lilliefors significance level for testing normality, was applied to continuous variables. Logarithmic or square root transformations were applied as needed to ensure normal distribution of the variables. The influence of the TNFRSF1B genotype or patient or control status, on sTNFR2 levels, was evaluated using a full-factorial general linear model. Because previous reports indicate the influence of obesity (15) and age (16) on serum sTNFR2 levels, BMI, and age were included in the model as covariates. Unpaired t test, or one-way ANOVA followed by the least significant difference test for post hoc
multiple mean comparisons, was used to compare the central tendencies of the different groups. To evaluate the association between discontinuous variables, we used the $\chi^2$ test. Because our alternate hypothesis was that certain alleles of the TNFRSF1B variants studied here (previously known to increase sTNFR2 levels) were associated with hyperandrogenism, one-tailed $\chi^2$ tests were used. The difference in the proportions among the groups and the 90% confidence interval (CI) of this difference were calculated. The 90% confidence limits used here are consistent with a one-tailed test using $\alpha = 0.05$ (32). Power analysis was performed using the G*Power software. An $\alpha$ value of 0.05 was chosen as the level of significance.

**Results**

**Serum sTNFR2 levels**

A full-factorial univariate general linear model was used to evaluate the influence on sTNFR2 levels of the different TNFRSF1B polymorphisms and patient or control status, introducing age and BMI as covariates. For this analysis, only subjects in whom all the polymorphisms had been studied were included. Therefore, the analysis included 76 patients and 30 controls from the Spanish sample. Because one previous study has shown that big (CA15/CA16) alleles of the CA-repeat polymorphism in intron 4 are associated with decreased sTNFR2 levels, compared with small (CA13/CA14) alleles (17), this polymorphism was analyzed after assigning the subjects to three different groups: subjects having two small alleles, subjects having two big alleles, and subjects carrying big and small alleles.

The model was statistically significant ($R^2 = 0.962$, $F = 34.22$, $P < 0.0001$), and showed that only the interaction between the 1663 G→A and the 1668 T→G variants in the 3′-UTR of TNFRSF1B ($P < 0.05$) induced changes in sTNFR2 concentrations, whereas patient or control status, the CA-repeat polymorphism in intron 4, the M196R polymorphism in exon 6, or any other interaction between these variables, did not influence sTNFR2 levels significantly (data not shown). The presence of a G allele at 1663 resulted in a marked increase in sTNFR2 levels in subjects homozygous for A alleles at 1663, compared with carriers of G alleles at this position (Table 1).

The only statistically significant covariant in the model was the BMI ($P = 0.019$), whereas age was not significant. Accordingly, serum sTNFR2 levels were higher in obese subjects (BMI > 25 kg/m²), compared with lean individuals (2226 ± 560 vs. 1890 ± 464 pg/ml, respectively, $P < 0.005$), but no correlation was observed between age and sTNFR2 levels ($r = 0.038$, $P = 0.638$).

**Association of TNFRSF1B genotypes and hyperandrogenism**

Initial studies included 103 hyperandrogenic patients (42 of them presenting with PCOS) and 36 controls from Spain, although some of the subjects were not included in all the molecular genetic analyses because of DNA exhaustion.

The 3′-UTR (exon 10) variants 1663 G→A, 1668 T→G, and 1690 T→C were not associated with hyperandrogenism (Table 2). The M196R variant and the CA-repeat microsatellite polymorphism in intron 4 of TNFRSF1B were in strong but not complete linkage disequilibrium: CA13/CA14 alleles were linked to 196R alleles, and CA15/CA16 alleles were linked to 196M alleles ($\chi^2 = 170$, $P < 0.00001$).

The sTNFR2 levels have been reported to be increased in obesity and to correlate with indexes of insulin resistance (11–14), conditions frequently associated with hyperandrogenism. Because a previous study associated the CA15/CA16/196M genotype with decreased serum sTNFR2 levels (17), our hypothesis was a possible association of the CA13/CA14 and 196R alleles with increased sTNFR2, hyperandrogenism, and insulin resistance. As stated above, neither 196R alleles nor the CA-repeat microsatellite polymorphism in intron 4 of TNFRSF1B influenced sTNFR2 levels, and we did not find association of any CA allele with hyperandrogenism (data not shown). However, 196R alleles appeared to be associated with hyperandrogenism and PCOS.

Hyperandrogenic patients presented with a 25.3% of 196R alleles, compared with 15.3% in controls (difference in proportion 10%, CI: -0.5–18.1%, $\chi^2 = 2.994$, $P = 0.056$). Because this result was close to statistical significance, we calculated the statistical power of the analysis, which was 0.41, meaning a 59% probability of excluding an association that was actually present in the population studied because of the small sample size (type 2 error).

**Table 2.** Single nucleotide polymorphisms in the 3′-UTR of TNFRSF1B and hyperandrogenism

<table>
<thead>
<tr>
<th>Patients n (%)</th>
<th>Controls n (%)</th>
<th>$\chi^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1663 G→A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>10 (27.8)</td>
<td>25 (26.3)</td>
<td>0.762</td>
</tr>
<tr>
<td>GA</td>
<td>15 (41.7)</td>
<td>47 (49.5)</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>11 (30.6)</td>
<td>23 (24.2)</td>
<td></td>
</tr>
<tr>
<td>1668 T→G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>31 (86.1)</td>
<td>80 (85.1)</td>
<td>0.021</td>
</tr>
<tr>
<td>TG</td>
<td>5 (13.9)</td>
<td>14 (14.6)</td>
<td></td>
</tr>
<tr>
<td>1690 T→C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>20 (55.6)</td>
<td>49 (52.1)</td>
<td>3.398</td>
</tr>
<tr>
<td>TC</td>
<td>10 (27.8)</td>
<td>38 (40.4)</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>6 (16.7)</td>
<td>7 (7.4)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Fisher’s exact test was used to calculate statistical significance for 2 × 2 tables.

**Table 1.** Effect of the 1663 G→A and 1668 T→G variants in the 3′-UTR of TNFRSF1B on serum sTNFR2 concentrations (picograms per milliliter) in 76 patients and 30 controls from Spain considered as a whole

<table>
<thead>
<tr>
<th>TNFRSF1B alleles</th>
<th>T1668G</th>
<th>T1668GT</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1663G</td>
<td>2162 ± 458 (32)</td>
<td>(0)</td>
<td>2162 ± 458 (32)</td>
</tr>
<tr>
<td>G1663A</td>
<td>2022 ± 501 (45)</td>
<td>1564 ± 238 (5)</td>
<td>1975 ± 499 (50)</td>
</tr>
<tr>
<td>A1663A</td>
<td>2111 ± 606 (15)</td>
<td>2460 ± 980 (9)</td>
<td>2242 ± 766 (24)</td>
</tr>
<tr>
<td>Total</td>
<td>2085 ± 503 (92)</td>
<td>2140 ± 898 (14)</td>
<td>2092 ± 565 (106)</td>
</tr>
</tbody>
</table>

Data are means ± SD. Values in parentheses indicate the number of subjects. According to the general linear model, the interaction between the 1663 G→A and 1668 T→G variants in the 3′-UTR of TNFRSF1B on sTNFR2 levels was significant with $P = 0.049$. On the contrary, the 1663 G→A ($P = 0.246$) and 1668 T→G ($P = 0.729$) variants did not influence sTNFR2 levels independently.
To increase the statistical power, we extended the analysis by including another 11 patients from Spain and 64 patients and 29 controls from Italy, confirming the association between 196R alleles of TNFRSF1B and hyperandrogenic disorders: Considering overall allele frequencies, hyperandrogenic patients presented with a 25.6% of 196R alleles, compared with 17.7% in controls (difference in proportions 7.9%, CI: 2–14.5%, $\chi^2 = 3.274, P = 0.044$). Furthermore, considering the genotype distribution 44.8% of hyperandrogenic patients were homo- or heterozygous for 196R alleles, compared with only 30.8% of controls (difference in proportions 14.0%, CI: 1.2–25.5%, $\chi^2 = 3.823, P = 0.034$).

Finally, scanning for other mutations in exon 6 did not reveal other abnormalities.

**Association of TNFRSF1B genotypes in the subgroup of PCOS patients**

At present, there is substantial ongoing debate regarding the most appropriate phenotype to study the possible influence of genetic factors in the pathogenesis of hyperandrogenic disorders, considering the heterogeneity of this condition. Because reputed authors in the field suggest that PCOS may be a separate entity in terms of inheritance, we have studied separately the distribution of TNFRSF1B alleles in the subgroup of patients with PCOS diagnosis, compared with the healthy controls.

Considering overall allele frequencies for the M196R variant, in the initial study including only Spanish patients, women presenting with PCOS carried 196R alleles more frequently than control women (30.3% in PCOS vs. 15.3% in controls, difference in proportions 15.0%, CI: 2.3–25.9%, $\chi^2 = 4.692, P = 0.024$). This result was confirmed in the extended study that also included Italian patients because 196R alleles were carried by 28.4% of PCOS patients, compared with 17.7% of healthy controls (difference in proportions 10.7%, CI: 2.2–18.2%, $\chi^2 = 4.960, P = 0.017$). Moreover, considering the genotype distribution 48.1% of PCOS patients were homo- or heterozygous for 196R alleles, compared with only 30.8% of controls (difference in proportions 17.3%, CI: 3.3–30.2%, $\chi^2 = 4.938, P = 0.019$).

**Lack of influence of TNFRSF1B on clinical and biochemical characteristics**

Patients were younger than controls, but both groups were matched for BMI (Table 3). Patients presented with increased hirsutism scores, serum total T, and free T, A, DHEAS, and insulin levels, whereas SHBG and fasting glucose were decreased, compared with controls (Table 3). The FIRI tended to be higher in patients than in controls (Table 3), reaching statistical significance when restricting the analysis to PCOS patients ($2.73 \pm 1.98$ in PCOS patients vs. $2.08 \pm 1.29$ in controls, $t = 2.525, P < 0.02$).

Because the M196R variant was associated with hyperandrogenism and PCOS and to avoid ascertainment bias favoring higher androgen levels in carriers of 196R alleles, the possible influence of the M196R variant on clinical and biochemical variables was studied separately in the control group and patients. No differences were observed in any of the variables studied (Table 4).

Finally, neither the CA-repeat polymorphism in intron 4 nor any of the three variants in the 3′-UTR of TNFRSF1B studied here influenced any of the clinical and biochemical characteristics mentioned above, including BMI and the FIRI (data not shown).

**Discussion**

Hyperandrogenism and PCOS are complex disorders in terms of inheritance, suggesting that multiple genes are involved in their pathogenesis. Several genes, including those encoding for steroidogenic enzymes and genes related to insulin secretion and action, have been proposed to play a role in the pathogenesis of PCOS and hyperandrogenism (33, 34). However, the search for contributing genes continues, especially because some of these associations have not been widely confirmed by later studies (24–26).

The relationship between hyperandrogenism and disorders of glucose tolerance is clearly established (6). The etiologic link between these conditions seems to be insulin resistance, which results in hyperinsulinemia and insulin-mediated stimulation of ovarian, and possibly adrenal, androgen secretion (6, 35, 36). Recently, inflammatory cytokines such as TNFa have been

**TABLE 3. Clinical and biochemical characteristic of hyperandrogenic patients and controls from Spain and Italy**

<table>
<thead>
<tr>
<th></th>
<th>Hyperandrogenic patients (n = 178)</th>
<th>Healthy controls (n = 65)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>$23.5 \pm 6.5$</td>
<td>$28.6 \pm 7.4$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>$26.6 \pm 7.1$</td>
<td>$27.5 \pm 7.8$</td>
<td>0.389</td>
</tr>
<tr>
<td>Hirsutism score</td>
<td>$14.2 \pm 5.6$</td>
<td>$1.5 \pm 1.5$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total testosterone</td>
<td>$2.2 \pm 1.0$</td>
<td>$1.4 \pm 0.5$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SHBG (nmol/liter)</td>
<td>$36 \pm 20$</td>
<td>$54 \pm 27$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Free T (pmol/liter)</td>
<td>$43 \pm 28$</td>
<td>$19 \pm 8.0$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>A (nmol/liter)</td>
<td>$10.3 \pm 4.7$</td>
<td>$8.7 \pm 3.1$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>DHEAS (µmol/liter)</td>
<td>$7.5 \pm 3.8$</td>
<td>$5.1 \pm 2.4$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Fasting insulin</td>
<td>$95 \pm 56$</td>
<td>$75 \pm 42$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>$4.5 \pm 0.5$</td>
<td>$4.7 \pm 0.6$</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>FIRI (mmol × mU × liter⁻²)</td>
<td>$2.53 \pm 1.68$</td>
<td>$2.07 \pm 1.29$</td>
<td>0.076</td>
</tr>
</tbody>
</table>

Data are means ± sd. Comparisons were made by the unpaired t test. The homogeneity of the variances was evaluated by the Levene’s test, and the results of the $t$ tests were interpreted accordingly.

* In 11 controls from Italian origin, serum samples were not available.

* Hirsutism scores were available for 154 patients and 36 controls, because in some patients and controls scores less than 5 were not recorded.
proposed to play a role in the pathogenesis of insulin resistance and related disorders (1). Certain high cytokine responder genotypes may have been selected during evolution because these genotypes provide survival advantage (1): 1) High cytokine levels facilitate defense against injury and infection; and 2) by preserving glucose for brain metabolism, insulin resistance could provide survival advantage against starvation during evolution, overcoming the possible inconveniences of atherosclerosis and glucose intolerance, which may appear with prolonged life expectancy of affected subjects, especially when these subjects are exposed to high carbohydrate and saturated fat contents, low-fiber diets, and sedentary habits.

Hyperandrogenism may also represent survival advantage and might also result from these high cytokine responder genotypes. The rapid maturation of the reproductive axis found in hyperandrogenic women, together with the increase in assertive behavior resulting from increased androgen secretion, might be advantageous during times of environmental stress (37–39). Also, the relative infertility of these women could increase the interval between pregnancies, decreasing the birth rate, and favoring maternal and infant survival (37). The possible involvement of high cytokine responder genotypes in hyperandrogenism is supported by the recent finding of increased levels of C-reactive protein in women with PCOS, indicating that low-grade chronic inflammation may underlay this disorder (40).

On the basis of these considerations, we recently decided to evaluate the possible role of the TNFα system in the pathogenesis of hyperandrogenism, and our results suggested that TNFα may be a contributing factor to the pathogenesis of hyperandrogenism (7).

To further explore the TNFα system, in the present study, we have evaluated the involvement of the TNFR2 system in hyperandrogenism. TNFR2 apparently mediates most of the metabolic actions of TNFα (41) and TNFα up-regulates markedly TNFα mRNA (42). Upon activation of TNFR2 by TNFα, sTNFR2 is shed into the circulation, modulating TNFα in a bimodal fashion depending on sTNFR2 levels: At low concentrations sTNFR2 preserves TNFα, but normalizes TNFα levels (43). Because TNFR2 mediates TNFα actions and TNFR2 acts as a modulator, TNFRSF1B can be considered as a candidate gene to explain TNFα-related disorders, including insulin resistance and the metabolic syndrome.

TNFRSF1B has been studied in several metabolic disorders. The 1690T→C variant has been described to influence BMI and insulin resistance (18), the CA-repeat/M196R polymorphisms influence serum lipid levels (16, 17, 31) and BMI and insulin resistance (18), the CA-repeat/M196R polymorphism is associated with hypertension (16) and familial combined hyperlipidemia (17). Moreover, the CA-repeat polymorphism has been recently proposed as a contributing factor to coronary artery disease (19).

Our present results suggest that the M196R polymorphism (which is in strong linkage disequilibrium with the CA-repeat polymorphism) is associated with hyperandrogenism because the frequency of carriers of the uncommon 196R allele was increased in hyperandrogenic patients, compared with healthy women. This association was stronger when restricting the analysis to the subset of women presenting with PCOS. Interestingly, consistent findings were obtained when considering as a whole two different population samples from Spain and northeastern Italy, suggesting that the association of the M196R polymorphism with hyperandrogenism is not limited to Spanish women. However, our present results do not provide any insight on the mechanism underlying this association, as occurred with previous studies regarding TNFRSF1B in other insulin-resistant disorders (16–19, 31).

At present there are very few data on the consequences of the M196R variant on TNFR2 function; therefore, the association with hyperandrogenism and PCOS should be considered with caution. Apparently, the M196R polymorphism does not result in significant conformational changes in the protein or in the expression of TNFRSF1B (44), despite the

### TABLE 4. Influence of the M196R (676 T → G) polymorphism on clinical and biochemical variables in healthy controls and hyperandrogenic women, from Spain and Italy

<table>
<thead>
<tr>
<th></th>
<th>Healthy controls</th>
<th>Whole group of hyperandrogenic patients</th>
<th>Subgroup of PCOS patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M196M (n = 45)</td>
<td>M196R and R196R (n = 20)</td>
<td>M196M (n = 95)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>28.4 ± 7.3</td>
<td>28.9 ± 7.8</td>
<td>22.8 ± 6.1</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.9 ± 8.2</td>
<td>26.8 ± 7.4</td>
<td>26.0 ± 6.3</td>
</tr>
<tr>
<td>Hirsutism scoreb</td>
<td>1.6 ± 1.7</td>
<td>1.4 ± 1.2</td>
<td>16.4 ± 5.7</td>
</tr>
<tr>
<td>Total testosterone (nmol/liter)</td>
<td>1.2 ± 0.5</td>
<td>1.4 ± 0.5</td>
<td>2.2 ± 1.1</td>
</tr>
<tr>
<td>SHBG (nmol/liter)</td>
<td>52 ± 25</td>
<td>49 ± 17</td>
<td>35 ± 21</td>
</tr>
<tr>
<td>Free T (pmol/liter)</td>
<td>19 ± 8</td>
<td>20 ± 8</td>
<td>44 ± 33</td>
</tr>
<tr>
<td>A (nmol/liter)</td>
<td>8.7 ± 3.1</td>
<td>9.1 ± 2.4</td>
<td>11.0 ± 4.4</td>
</tr>
<tr>
<td>DHEAS (μmol/liter)</td>
<td>4.7 ± 2.2</td>
<td>6.0 ± 2.8</td>
<td>7.4 ± 5.4</td>
</tr>
<tr>
<td>Fasting insulin (pmol/liter)</td>
<td>77 ± 55</td>
<td>66 ± 31</td>
<td>94 ± 60</td>
</tr>
<tr>
<td>Fasting glucose (nmol/liter)</td>
<td>4.7 ± 0.6</td>
<td>4.8 ± 0.7</td>
<td>4.5 ± 0.6</td>
</tr>
<tr>
<td>FRI (μmol × mU × liter⁻²)</td>
<td>2.11 ± 1.38</td>
<td>1.90 ± 1.07</td>
<td>2.50 ± 1.78</td>
</tr>
</tbody>
</table>

Data are means ± SD. Patients and controls were analyzed separately. No significant differences between genotypes were found using unpaired t test. The homogeneity of the variances was evaluated by the Levene’s test, and the results of the t tests were interpreted accordingly.

b Hirsutism scores were available for 26 controls and 85 patients presenting with the M196M genotype, and in 10 controls and 63 patients presenting with the M196R or R196R genotype, because in 29 controls and 25 PCOS patients scores below 5 were not recorded.
fact that exon 6 encodes the proteolytic site that mediates shedding of sTNFR2. However, because of the complex functional interdependency among TNFα, TNFR2, and sTNFR2, the possibility that minor changes in TNFR2 structure or function induced by the M196R variant might influence TNFα actions in vivo exists.

Both the M196R variant in exon 6 and the CA-repeat polymorphism in intron 4 of \textit{TNFRSF1B} have been shown to influence shedding of TNFR2 in two previous studies (16, 17), although the results were not completely concordant. In our present study, however, the only genotypic effect of \textit{TNFRSF1B} on sTNFR2 levels is an interaction between the 1663 G→A and the 1668 T→G variants, but no effect of the M196R variant and the CA-repeat polymorphism on sTNFR2 levels was found. This discrepancy with the previous studies cited above (16, 17) might be due to the peculiar characteristics of subjects we studied (i.e. hyperandrogenic women). Alternatively, it could depend on the different statistical approach used in our study. The use of a more strict general linear model allowed us to correct for age and obesity, avoiding the confounding interference of these factors on the possible influence of the \textit{TNFRSF1B} genotype, and patient or control status, on sTNFR2 levels. In addition to avoiding spurious associations, this approach allowed us to explore precisely the interaction among the different genomic variants described here, between them and with hyperandrogenism.

However, the association between 196R alleles and hyperandrogenism should be weighted carefully because carriers of this variant did not present with increased serum androgens, hirsutism score, BMI, or markers of insulin resistance, compared with subjects homozygous for 196M alleles. This finding suggests that the \textit{TNFRSF1B} genotype might be a contributing factor in the pathogenesis of hyperandrogenism by a hypothetical facilitation of TNFα actions (7), but there exists the possibility that the M196R variant is in linkage disequilibrium with yet unknown mutation, which is the actual contributor to hyperandrogenism, as suggested for familial combined hyperlipidemia.

As stated above, carriers of −308A TNFα alleles presented with higher androgen levels, compared with wild-type alleles (7). Interestingly, we have not found any association between the 196R alleles of \textit{TNFRSF1B} and −308A alleles of the TNFα gene in 79 subjects who had been previously genotyped for the TNFα G-308A variant (χ² = 0.202, \( P = 0.904 \)), ruling out any significant cooperative effect of these genomic variants on the pathogenesis of androgen excess.

In summary, our present results suggest that the \textit{TNFRSF1B} genotype might influence hyperandrogenism and PCOS, further supporting the contribution of inflammatory cytokines to the pathogenesis of these disorders.

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References