A Single Nucleotide Polymorphism in STK11 Influences Insulin Sensitivity and Metformin Efficacy in Hyperinsulinemic Girls With Androgen Excess

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Objective — Serine-threonine kinase STK11 catalyzes the AMP-activated protein kinase complex. We tested the hypothesis that a gene variant in STK11 contributes to variation in insulin sensitivity and metformin efficacy.

Research Design and Methods — We studied the effects of a single nucleotide polymorphism (SNP) (rs8111699) in STK11 on endocrine-metabolic and body composition indexes before and after 1 year of metformin in 85 hyperinsulinemic girls with androgen excess, representing a continuum from prepuberal girls with a combined history of low birth weight and precocious pubarche over to postmenarchial girls with hyperinsulinemic ovarian hyperandrogenism. Metformin was dosed at 425 mg/day in younger girls and 850 mg/day in older girls. STK11 rs8111699 was genotyped. Endocrine-metabolic features were assessed in the fasting state; body composition was estimated by absorptiometry.

Results — Genotype effects were similar in younger and older girls. At baseline, the mutated G allele in STK11 rs8111699 was associated with higher insulin and IGF-I levels (both P < 0.005). The response to metformin differed by STK11 genotype: GG homozygotes (n = 24) had robust metabolic improvements, GC heterozygotes (n = 38) had intermediate responses, and CC homozygotes (n = 23) had almost no response. Such differences were found for 1-year changes in body composition, circulating insulin, IGF-I, free androgen index, and lipids (all P < 0.005).

Conclusions — In hyperinsulinemic girls with androgen excess, the STK11 rs8111699 SNP influences insulin sensitivity and metformin efficacy, so that the girls with the least favorable endocrine-metabolic profile improve most with metformin therapy.

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Defects in STK11 expression contribute to interindividual differences in metformin efficacy.

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The actions of metformin seem to be largely exerted through activation of AMP-activated protein kinase (AMPK), a conserved regulator of the cellular response to low energy, in many organs, including liver and skeletal muscle (10,11). The activation of AMPK in the liver is catalyzed by serine-threonine kinase (STK11, formerly known as LKB1), a tumor suppressor gene defective in Peutz-Jeghers syndrome (12); deletion of hepatic STK11 in mice results in a nearly complete loss of AMPK activity, leading to adipogenesis and lipogenic gene expression (13). STK11 serves as a mediator of metformin effects, rather than as a direct target of metformin (14).

Recently, the C allele of a single nucleotide polymorphism (SNP) (rs8111699) in STK11 has been associated with a reduced ovulatory response to metformin in women with PCOS (15). In a pilot study, we have tested the hypothesis that the same SNP in STK11 also influences the endocrine-metabolic and body composition changes after metformin therapy in girls with hyperinsulinemic androgen excess.
birth) and precocious pubarche (appearance of pubic hair before age 8 years) attributable to an exaggerated adrenarche, based on high serum levels of dehydroepiandrosterone sulfate or androstenedione at diagnosis of precocious pubarche (8). The girls were, in addition, hyperinsulinemic, as judged by the insulin response after an oral glucose tolerance test (16). All of these subjects participated in a controlled long-term study exploring the effects of early metformin treatment (ISRCTN84749320); more specifically, they form together the study subgroup that was initially randomly assigned to receive metformin for at least 1 year (425 mg, daily at dinner time) (9).

The early postmenarcheal girls (n = 31; aged 12.2 ± 0.1 years; BMI 21.5 ± 0.5 kg/m²) manifested biochemical androgen excess characterized as the ensemble of 1) absence of clinical signs or symptoms of androgen excess (17) and 2) hyperinsulinemia (oral glucose tolerance test) and 3) ovarian androgen excess suggested by increased testosterone levels and confirmed by 17-hydroxyprogesterone hyperresponse to gonadotropin-releasing hormone agonist (17,18). The girls in the present analysis were enrolled in clinical studies that were initiated before 2003, thus before compulsory trial registration; as described, all these girls had a history of LBW-preocious pubarche and were already in the early postmenarcheal phase (6–12 months beyond menarche) at start of metformin intervention (8,19).

The adolescent girls (n = 36; aged 15.5 ± 0.3 years; BMI 21.9 ± 0.5 kg/m²) were consecutive patients with PCOS who were at least 2 years beyond menarche and who were followed in the outpatient clinic according to standard procedures, including yearly blood sampling in the fasting state (once with DNA extraction) and yearly assessment of body composition. The inclusion criteria were the same as those for the early postmenarcheal girls, except that the presence of clinical markers of androgen excess was now required: hirsutism (Ferriman-Gallwey score >8) plus either amenorrhea (menses absent for >3 months) or oligomenorrhea (menstrual cycle >45 days). In all postmenarcheal girls, baseline assessments were performed in the follicular phase (day 3–7) or after 2 months of amenorrhea.

All postmenarcheal girls received metformin for at least 1 year in monotherapy (850 mg, daily at dinner time). At the start of metformin treatment, none of the 85 girls presented evidence for thyroid dysfunction, Cushing syndrome, hyperprolactinemia, glucose intolerance, or late-onset adrenal hyperplasia; for at least 6 months before the start of metformin, none of the girls received an estroprogestagen or other medication known to affect gonadal function or carbohydrate metabolism.

**Clinical, endocrine, and metabolic assessments**

Height and weight were measured, and BMI was calculated (weight in kilograms divided by the square of height in meters). BMI standard deviation scores (SDSs) were derived from regional normative data (20).

Fasting blood glucose, serum insulin, IGF-1, sex hormone–binding globulin (SHBG), and testosterone were measured as described previously (9). Free androgen index was calculated as testosterone × 100/SHBG. Serum lipids were measured by routine laboratory methods.

**Genetic analysis**

Genomic DNA was purified from whole blood samples using commercial reagents (Genta Puragene Cell Kit; Qiagen Iberia, Madrid, Spain). The STK11 rs8111699 SNP was genotyped by a fluorescent genotyping system (KASPar SNP Genotyping System; KBiosciences, Hoddesdon, U.K.). In brief, 10 pg genomic DNA was incubated in a 8-μl solution of a combined mix containing 100 mol/l allele specific primer 1 (FAM, x-axis, allele G), 5′-GAGGTGACCAAGTTCTACGTCACTGTTCAGAAAGCAG-3′, 100 mol/l allele-specific primer 2 (VIC, y-axis, allele C), 5′-GGTTACGCGATCCGGATTCACCTGACTGTTCAGAAAGCAG-3′, 100 mol/l common reverse primer 5′-GGTGAAGCCTGACTGTTCAGAAAGCAG-3′, 100 mol/l common reverse primer 5′-GGTTACGCGATCCGGATTCACCTGACTGTTCAGAAAGCAG-3′, 100 mol/l Tris-HCl, pH 8.3, KTaq polymerase, 1.8 mmol/l MgCl₂, and ROX as a passive reference. The PCR mix was incubated at 94°C for 15 min, followed by 20 cycles of 94°C for 10 s, 57°C for 5 s, and 72°C for 10 s. Thereafter, the PCR mix was incubated for 18 cycles at 94°C for 10 s, 57°C for 20 s, and 72°C for 40 s. Samples were then read in a fluorescence plate reader. The KASPar system uses the fluoros FAM and VIC for distinguishing among genotypes and ROX as a passive reference. The corresponding genotypes for this SNP (CC vs. CG vs. GG) did not differ among prepuberty (33% vs. 44% vs. 22%), early postmenarche (26% vs. 49% vs. 26%), and PCOS (25% vs. 42% vs. 33%) subjects in our study (P = 0.89). The distributions were also tested for Hardy-Weinberg equilibrium, and none deviated significantly.

**Body composition**

Body composition was assessed by dual-energy X-ray absorptiometry with a LunarProdigy and Lunar software (version 3.4/3.5; Lunar, Madison, WI) (9). Total irradiation dose per assessment was 0.1 mSv; coefficients of variation for scanning precision are 2.2 and 2.6% for fat and lean mass (8,9,19).

**Ethics and statistics**

The study protocol was approved by the Institutional Review Board of Hospital of Sant Joan de Déu. Informed consent was obtained from the patients and/or from parents, and assent was obtained from girls, as appropriate. The clinical, biochemical, and body composition data from prepubertal and early postmenarcheal subjects have been reported separately (8,9,19). Statistical analyses were performed using SPSS (version 12.0; SPSS, Chicago, IL). Quantitative phenotypic data were compared across genotypes by a repeated-measures general linear model, computing the genotype as the between-subjects effect and both baseline and 1-year values as the within-subjects effect. Differences in 1-year changes across genotypes were tested by the interaction term among the between- and within-subjects effects. P < 0.05 was considered statistically significant.

**RESULTS** — Clinical and laboratory characteristics of the study subjects are shown in Table 1. Genotype effects were similar in prepubertal, early postmenarcheal, and PCOS subjects, and therefore genotype-phenotype associations are presented for all 85 girls studied as a single group (adjusted for group allocation) to maximize the statistical power.

At diagnosis, the G allele in STK11 rs8111699 was associated with a poorer metabolic profile, specifically with higher insulin and IGF-I (both P < 0.005) (Table 2). The presence of this allele was at baseline unrelated to age, weight, body composition parameters, and serum androgens and lipids (Table 2).
STK11 and metformin efficacy

Table 1—Clinical, biochemical, and body composition variables before and after 1 year of metformin in the study subjects

|                      | All          | Prepubertal | Early postmenarcheal | PCOS         |
|----------------------|--------------|-------------|----------------------|--------------|
|                      | Baseline     | 1 year of metformin | Baseline     | 1 year of metformin | Baseline     | 1 year of metformin |
|                      | n           | Age (years) | BMI (kg/m²) | BMI SDS | Glucose (mg/dl) | Insulin (mIU/l) | IGF-I (ng/ml) | SHBG (µg/dl) | Testosterone (ng/dl) | Free androgen index | Triglycerides (mg/dl) | LDL cholesterol (mg/dl) | HDL cholesterol (mg/dl) | LDL cholesterol-to-HDL cholesterol ratio | Fat mass (kg) | Abdominal fat (kg) | Lean mass (kg) |
|                      | 85          | 12.6 ± 0.3 | 21.2 ± 0.3 | 0.8 ± 0.1 | 88 ± 1 | 12.7 ± 0.5 | 286 ± 10 | 1.1 ± 0.1 | 73 ± 5 | 8.7 ± 0.8 | 79 ± 4 | 99 ± 3 | 56 ± 1 | 1.9 ± 0.1 | 17.1 ± 0.8 | 5.1 ± 0.3 | 31.2 ± 0.7 |
|                      | 85          | 18         | 21.4 ± 0.3 | 0.9 ± 1.1 | 88 ± 1 | 9.6 ± 1.1 | 254 ± 7† | 1.3 ± 0.1 | 52 ± 3† | 4.6 ± 0.3† | 64 ± 3† | 89 ± 2† | 62 ± 1† | 1.5 ± 0.1† | 16.8 ± 0.8 | 4.7 ± 0.3† | 3.0 ± 0.7† |
|                      | 18          | 18         | 19.3 ± 0.6 | 1.5 ± 0.3 | 88 ± 2 | 9.3 ± 0.9 | 219 ± 12 | 1.5 ± 0.1 | 35 ± 4 | 2.5 ± 0.4* | 70 ± 11 | 108 ± 7 | 60 ± 3 | 2.0 ± 0.2 | 12.0 ± 0.9 | 3.5 ± 0.4 | 23.0 ± 0.9† |
|                      | 18          | —          | 19.7 ± 0.6 | 1.5 ± 0.4 | 91 ± 2 | 10.4 ± 0.7 | 283 ± 13† | 1.5 ± 0.1 | 30 ± 4 | 2.5 ± 0.4* | 62 ± 3† | 87 ± 3 | 59 ± 2* | 1.7 ± 0.1 | 4.7 ± 0.4† | 3.5 ± 0.4 | 23.0 ± 0.9† |
|                      | 31          | —          | 21.5 ± 0.5 | 0.6 ± 0.3 | 89 ± 1 | 13.2 ± 0.7 | 283 ± 13† | 1.1 ± 0.1 | 71 ± 5 | 4.7 ± 0.4† | 62 ± 3† | 38 ± 3 | 59 ± 2* | 1.5 ± 0.1 | 1.3 ± 0.1 | 4.7 ± 0.4† | 3.5 ± 0.4 | 23.0 ± 0.9† |
|                      | 31          | —          | 21.7 ± 0.5 | 0.7 ± 0.4 | 90 ± 1 | 10.4 ± 0.7 | 283 ± 13† | 1.3 ± 0.1 | 52 ± 3* | 4.7 ± 0.4† | 62 ± 3† | 39 ± 3 | 62 ± 2* | 1.5 ± 0.1 | 1.3 ± 0.1 | 4.7 ± 0.4† | 3.5 ± 0.4 | 23.0 ± 0.9† |
|                      | 36          | —          | 21.9 ± 0.5 | 0.8 ± 0.3 | 87 ± 1 | 13.8 ± 0.9 | 287 ± 10 | 1.0 ± 0.1 | 94 ± 8 | 5.6 ± 0.6 | 62 ± 4* | 84 ± 3 | 66 ± 2† | 1.5 ± 0.1 | 1.2 ± 0.1 | 5.6 ± 0.6 | 62 ± 4* | 84 ± 3 |

Values are means ± SEM. Values in healthy girls matched for BMI: prepubertal (n = 24; aged 8.3 ± 0.3 years): insulin 65 ± 0.5 mIU/l, IGF-I 200 ± 15 ng/ml, SHBG 3.6 ± 0.1 µg/dl, testosterone 16 ± 2 ng/dl; pubertal (n = 24; age 15.3 ± 0.2 years): insulin 8.8 ± 0.4 mIU/l, IGF-I 384 ± 26 ng/ml, SHBG 1.9 ± 0.1 µg/dl, testosterone 31 ± 3 ng/dl. Lipid profile: triglycerides 55 ± 4 mg/dl; LDL 83 ± 3 mg/dl; HDL 65 ± 2 mg/dl (8, 24 and Iñáñez et al.; JCEM 2004; 89, 433–337). P values are from paired-samples Student t-test. *P < 0.005; †P < 0.0001.

After 1 year of therapy, there were pronounced differences in the response to metformin according to the STK11 rs8111699 genotype, as reflected by a robust response in the main endocrine-metabolic and body composition parameters in GG homozygotes, with an intermediate response in CG heterozygotes and almost no response in CC homozygotes (Table 2). Such differences were found for 1-year changes in body composition, circulating insulin, IGF-I, free androgen index, and lipids (all P < 0.005; Table 2). All of these comparisons were adjusted for differences in baseline insulin levels.

CONCLUSIONS — Our results indicate that the STK11 rs8111699 SNP influences both insulin sensitivity and metformin efficacy in hyperinsulinemic girls with androgen excess. Despite the fact that neither STK11 nor AMPK is a direct target of metformin (14), both are necessary for metformin actions (14). It has been proposed that STK11 is constitutively active and that metformin exerts its hypoglycemic effects through a modification in AMPK, rendering it a better substrate for STK11 (10). Although the role of the studied intronic SNP in STK11 has yet to be defined, it is known that intronic SNPs may regulate gene expression and alternative splicing (21). The STK11 rs8111699 SNP may also be in linkage disequilibrium with an active SNP in the kinase gene region.

The STK11 rs8111699 SNP was associated with baseline insulin sensitivity in girls with hyperinsulinemic androgen excess. Bearing in mind that STK11 may be constitutively active, we postulate that carrying the G allele in the STK11 rs8111699 SNP can result in lower STK11 activity and therefore lower efficiency of AMPK phosphorylation by STK11.

The same G allele in the STK11 rs8111699 SNP was associated with pronounced changes in endocrine-metabolic and body composition parameters upon metformin treatment, effects that were nearly absent in C homozygous subjects. We suggest that metformin restores the efficiency of AMPK phosphorylation by STK11 in carriers of the G allele and has less or no effect in the absence of such allele. Our observations concur with those of Legro et al. (15), who found a stepwise increase in ovulation rate from C/C to C/G to G/G genotype in women with PCOS treated with metformin.

AMPK is an indirect target of a number of drugs besides metformin. These include the commonly used thiazolidinediones and statins (22). The STK11 rs8111699 SNP may thus play a broader role in metabolic and cardiovascular diseases by modulating the baseline condition as well as the response to treatment with the above-mentioned pharmacological agents.

A limitation of our study is the insufficient power to analyze the effect of the STK11 SNP in each of the three subgroups within the total study population. Such an analysis would have been of interest because each of the consecutive subgroups is thought to represent a developmental stage of hyperinsulinemic androgen excess. It should be noted, however, that the results remained essentially the same with and without adjustment for subgroup allocation in the multivariate model.

Hyperinsulinemic androgen excess is a rather heterogeneous condition (3). The identification of genetic polymorphisms that contribute to predict the response to commonly prescribed medications should assist clinicians in their selection.
Clinical, biochemical, and body composition variables before and after 1 year on metformin according to STK11 rs8111699 genotype in the study subjects

| Genotype  | Testosterone (ng/dl) | Insulin (mIU/l) | IGF-I (ng/ml) | BMI (kg/m²) | WC (cm) | Age (years) | P value |
|-----------|----------------------|-----------------|---------------|-------------|---------|-------------|---------|
| CC        | 77 ± 26              | 10.8 ± 3.9      | 251 ± 13.9    | 12.0 ± 0.8  | 92 ± 3.5 | 15.3 ± 0.7  | 0.0004  |
| GC        | 77 ± 26              | 10.8 ± 3.9      | 251 ± 13.9    | 12.0 ± 0.8  | 92 ± 3.5 | 15.3 ± 0.7  | 0.0004  |
| GG        | 77 ± 26              | 10.8 ± 3.9      | 251 ± 13.9    | 12.0 ± 0.8  | 92 ± 3.5 | 15.3 ± 0.7  | 0.0004  |

Baseline (mean ± SEM)
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