Metabolic and Hormonal Consequences of the „Obesity Risk“ MC4R Variant (rs12970134) in Czech Women

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Summary

Although the mutations in *MC4R* gene became known as the most common genetic cause of human obesity, the effect of rs12970134 A/G near *MC4R* gene on insulin resistance has been described. The aim of this study was to determine the effect of rs12970134 on obesity, hormone levels, and glucose metabolism in a cohort of women varying in glucose tolerance: 850 normoglycemic women, 423 diagnosed with polycystic ovary syndrome (PCOS), 402 gestational diabetics (GDM), and 250 type 2 diabetic (T2D) women. We did not confirm the explicit effect of rs12970134 on obesity. However, the influence of the A-allele on body adiposity index was observed in a cohort of women diagnosed with PCOS. In normoglycemic women, the A-allele carriership was associated with lower fasting levels of glucose, insulin, C-peptide, and index of insulin resistance. Furthermore, higher levels of growth hormone, leptin and SHBG, and lower levels of fT3, testosterone, and androstenedione were recorded in normoglycemic A-allele carriers. In conclusion, the study presents the evidence of the impact of rs12970134 on complex hypothalamic regulations.

Key words

MC4R variant (rs12970134) • Obesity • Glucose metabolism • Leptin • Growth hormone • Thyroid hormones

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Introduction

The *MC4R* (melanocortin-4 receptor) gene, encoding G-protein-coupled, seven- transmembrane receptor, is expressed predominantly in the brain. *MC4R* was the first locus described in association with dominantly inherited morbid human obesity and was the most common genetic cause of human obesity described before the era of genome wide association studies (Grant *et al.* 2009).

The melanocortins are involved in a variety of physiological processes, including pigmentation, steroidogenesis – especially glucocorticoid production, exocrine secretion, sexual function, analgesia, inflammation, immunomodulation or cardiovascular regulation. They have also important functions in the central and peripheral nervous systems including the regulation of energy homeostasis and body weight through its role in appetite and energy expenditure *via* leptin, ghrelin and agouti related protein, a potent endogenous antagonist of MC4R (Huszar *et al.* 1997, Yang *et al.* 1999).

The genetic defects of various steps of melanocortin signaling were studied very intensively and the five-year evidence, supporting their role in the control of appetite and body weight in humans, was summarized by O’Rahilly’s group (Yeo *et al.* 2000).

Human monogenic forms of obesity are linked to mutations in genes involved in the leptin/melanocortin axis. Homozygous mutations of leptin and leptin receptor gene causing hyperphagia and a severe early onset obesity associated with pituitary dysfunction were published (Farooqi *et al.* 2002,

Clement *et al.* 1998) as well as a phenotype of patient with complete absence of MC4R activity (Lubrano-Berthelier *et al.* 2004). Heterozygous mutations in *MC4R* are found in 2-6 % of severe obesity cases and they represent the most frequent genetic cause of human severe obesity (Vaisse *et al.* 2000, Dubern *et al.* 2001, Farooqi *et al.* 2003, Yeo *et al.* 2003, Hinney *et al.* 2003, Hainerová *et al.* 2007, Calton *et al.* 2009). However, the functional analysis of the mutant *MC4R* indicates that the receptor defects range from loss of function to constitutive activation and that the expressivity of *MC4R*-associated obesity is very variable (Vaisse *et al.* 2000).

Although previous studies have reported several rare *MC4R* mutations in the development of extreme and early-onset obesity, recent publications based on the genome-wide association studies (GWAS) have identified several common genetic polymorphisms near *MC4R* gene contributing to the common obesity (Loos *et al.* 2008, Chambers *et al.* 2008, Thorleifsson *et al.* 2009). Among these variants, the rs17782313 and rs12970134 were studied most often. However, the results have been inconsistent, esp. among East Asians and Africans. Although majority of studies showed significant association with obesity-related traits, several studies revealed non-significant association (Sherag *et al.* 2010, Xi *et al.* 2012a, Bazzi *et al.* 2014, Albuquerque *et al.* 2014, Fernandez *et al.* 2015).

Unclear remains also an influence of genetic variants of *MC4R* on glucose metabolism. Although an association of rs12970134 *MC4R* variant with insulin resistance was published (Chambers *et al.* 2008), it was not confirmed by other studies (Zobel *et al.* 2009, Kring *et al.* 2010, Bazzi *et al.* 2014).

We aimed to determine the prevalence of rs12970134 polymorphism in groups of thoroughly characterized Czech adult women differing in glucose tolerance and to study wide range of the anthropometric and metabolic consequences of the minor A-allele, with a special attention focused on glucose metabolism and insulin sensitivity.

Methods

Study subjects

The influence of the *MC4R* variant rs12970134 on anthropometric and biochemical parameters was evaluated in 850 women with normal glucose tolerance (median and upper and lower confidence limit of age was

30.4 [29.6; 31.3] years, BMI 22.8 [22.5; 23.3] kg/m², minimum: 16 kg/m²; maximum: 49.3 kg/m²).

To evaluate the influence of the SNP in women varying in glucose tolerance, it was assessed also in a group of 423 women (age: 27.6 [26.7; 28.2] years; BMI: 25.4 [24.7; 26.1] kg/m²) diagnosed with polycystic ovary syndrome (PCOS), disorder characterized by impaired glucose tolerance, in 402 women with a positive history of gestational diabetes mellitus (GDM) (age: 33.4 [32.9; 33.8] years; BMI: 23.1 [22.5; 23.6] kg/m²), and in 250 women diagnosed with type 2 diabetes mellitus (T2D) (age: 60.6 [59.8; 61.5] years; BMI: 31.1 [30.5; 32.9] kg/m²).

Anthropometric and biochemical characterization

Body weight, height, waist, abdominal and gluteal circumferences were measured. Anthropometric indices were calculated: body mass index (BMI)=weight [kg]/height [m]², waist-to-hip ratio (WHR) and body adiposity index (BAI)=(hip circumference [cm])/((height [m])^{1.5} - 18).

Oral glucose tolerance test (oGTT) with 75 g of glucose was performed in each woman except T2D group. Blood samples were taken before the beginning of the oGTT and then every 30 min during a period of 3 h. In this time points, glycemia, insulinemia, and C-peptide were evaluated. Furthermore, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triacylglycerols and glycated hemoglobine (HbA1c) were assessed [Cobas 6000, Roche Diagnostics]. Fasting levels of leptin [RIA; DRG], growth hormone, sex-hormone binding globuline (SHBG), insulin-like growth factor-1 (IGF-1) [IRMA; Beckman Coulter], glucagon [RIA; Eurodiagnostica], free triiodothyronine (fT3), free thyroxine (fT4), thyroid-stimulating hormone (TSH) [Cobas 6000; Roche Diagnostics], cortisol, testosterone and androstenedione [RIA; Beckman Coulter] were also measured.

Indices of glucose homeostasis (HOMAR= $I_0 \times G_0 / 22.5$; Quicki= $1 / (\log(\text{fasting insulin } \mu\text{U/ml}) + \log(\text{fasting glucose mg/dl}))$; HOMA $\beta = 20 \times I_0 / (G_0 - 3.5)$; insulinogenic index= $(I_{30} - I_0) / (G_{30} - G_0)$; Cederholm= $[75000 + (G_0 - G_{120}) \times 1.15 \times 180 \times 0.19 \times \text{weight}] / [120 \times G_{\text{mean}} \times \log(I_{\text{mean}})]$; Matsuda= $10^4 / \sqrt{(I_{\text{mean}} \times G_{\text{mean}} \times G_0 \times I_0)}$ and areas under curves (AUCs) of blood glucose, insulin, and C-peptide were calculated.

All women signed informed consent approved by Ethics Committee of the Institute of Endocrinology.

Genetic analysis

DNA was isolated from the peripheral blood using automatic device QuickGene 610L (FujiFilm Life Science, Japan) and commercial kit (QuickGene Whole Blood kit L, Kurabo Industries Ltd., Japan). Endpoint genotyping of the polymorphism rs12970134 near MC4R

was performed using TaqMan assays (Applied Biosystems, LightCycler 480 System, Roche).

Statistical analysis

For the comparison of the genotypic frequencies between the groups, a chi-square test was used. For the

Table 1. Associations of rs12970134 with anthropometric parameters in normoglycemic, PCOS, GDM and T2D women.

Normoglycemic (n=850)	GG (n=468)	AA+AG (n=382)	p
<i>Age (years)</i>	28.9(28.2;30.0)	29.6(28.3;30.5)	0.15
<i>Height (cm)</i>	168(167;168)	168(167;169)	0.38
<i>Abdominal circumference (cm)</i>	82.5(81.3;84.1)	81.5(80.2;83)	0.21
<i>Waist circumference (cm)</i>	75(74;76)	74(73;76)	0.51
<i>Gluteal circumference (cm)</i>	100(99;101)	100(99;101)	0.92
<i>BMI (kg/m²)</i>	23.1(22.5;23.5)	22.6 (22.3;23.3)	0.3
<i>BAI</i>	27.4(26.9;27.9)	27.4(26.8;28.1)	0.93
<i>WHR</i>	0.84(0.83;0.84)	0.83(0.81;0.84)	0.29
PCOS (n=423)	GG (n=253)	AA+AG (n=170)	p
<i>Age (years)</i>	27.0(26.2;28.3)	27.5(26.0;28.3)	0.69
<i>Height (cm)</i>	168(167;169)	167(166;169)	0.38
<i>Abdominal circumference (cm)</i>	87(84;89)	91(86;98)	0.07
<i>Waist circumference (cm)</i>	79(76;80)	84(80;88)	0.049
<i>Gluteal circumference (cm)</i>	101(100;103)	106(104;110)	0.01
<i>BMI (kg/m²)</i>	25.2(24.1;26.1)	26(24.7;28.1)	0.09
<i>BAI</i>	28.5(27.5;29.6)	31.1(29.4;33.0)	0.01
<i>WHR</i>	0.76(0.76;0.77)	0.79(0.76;0.81)	0.16
GDM (n=402)	GG (n=211)	AA+AG (n=191)	p
<i>Age (years)</i>	32.9(32;33.7)	33.3(32.3;34)	0.29
<i>Height (cm)</i>	167(166;168)	168(167;168)	0.91
<i>Abdominal circumference (cm)</i>	85(82.4;87.1)	85.4(83;88)	0.88
<i>Waist circumference (cm)</i>	75(74;77)	76(75;79)	0.65
<i>Gluteal circumference (cm)</i>	98(97;101)	98(97;101)	0.76
<i>BMI (kg/m²)</i>	23(22.4;23.8)	23.4(22.3;24.3)	0.49
<i>BAI</i>	27.4(26.8;28.6)	27.8(26.6;28.9)	0.66
<i>WHR</i>	0.77(0.76;0.79)	0.78(0.77;0.79)	0.55
T2D (n=250)	GG (n=120)	AA+AG (n=130)	p
<i>Age (years)</i>	60.6(59.1;62.2)	59.1(57.3;61.0)	0.58
<i>Height (cm)</i>	162(160;164)	160(159;162)	0.07
<i>Abdominal circumference (cm)</i>	108(102;111)	106(98;109)	0.72
<i>Waist circumference (cm)</i>	101(97;104)	100(99;104)	0.46
<i>Gluteal circumference (cm)</i>	113(111;116)	116(112;117)	0.74
<i>BMI (kg/m²)</i>	31.2(29.8;33.2)	31.3(30.5;32.4)	0.71
<i>BAI</i>	36.4(35.8;38.1)	37.8(37.1;39.3)	0.08
<i>WHR</i>	0.87(0.86;0.89)	0.87(0.86;0.89)	0.74

Levels are presented as a median (95 % LCL; 95 % UCL); Mann-Whitney test.

evaluation of the differences in anthropometric and biochemical parameters, a robust non-parametric Mann-Whitney test was used (NCSS, 2004). Statistical significance was set at $p < 0.05$.

Results

The allelic frequencies did not differ between groups ($\chi^2 = 4.99$; $p = 0.29$). Genotype frequencies of a whole cohort (GG/AG/AA: 55.7 % / 37.8 % / 6.5 %) were in Hardy-Weinberg equilibrium ($p = 0.95$), the minor A-allele frequency was 25 %.

In a cohort of normoglycemic women, no association of the polymorphism rs12970134 near *MC4R* with obesity or with obesity-related traits has been found (dominant model, Table 1). However, in a part of this cohort, in which questionnaire data were available, carriers of the minor A-allele had significantly higher maximal and minimal body weight achieved in adulthood (GG ($n = 183$) vs. AG+AA ($n = 169$): maximal 64 (62; 68) vs. 70 (65; 75) kg, $p = 0.04$; minimal 54 (52; 56) vs. 57 (55; 61) kg, $p = 0.01$, resp.).

Nevertheless, the influence of A-allele on obesity-related traits was clearly expressed in women diagnosed with PCOS. Carriers of the minor A-allele had significantly higher BAI ($p = 0.01$), waist ($p = 0.049$) and gluteal ($p = 0.01$) circumferences (Table 1). Further anthropometric parameters such as BMI and abdominal

circumference were also higher in carriers of the A-allele compared to non-carriers, although these findings did not achieve statistical significance. A trend towards higher BAI in A-allele carriers was observed also in the group of T2D women (Table 1).

An association with glucose metabolism has been found in the cohort of women with normal glucose tolerance (Table 2): carriers of the A-allele had lower fasting blood glucose ($p = 0.001$), insulin ($p = 0.005$) and C-peptide ($p = 0.038$), lower percentage of HbA1c ($p = 0.043$) and higher value of glucose to insulin ratio ($p = 0.035$) compared to non-carriers. No differences were found in stimulated parameters (AUCs). Carriers of the A-allele were more insulin sensitive – they had significantly lower value of insulin resistance index (HOMAR; $p = 0.002$) and higher value of insulin sensitivity index (Quicki; $p = 0.002$). However, indices of insulin sensitivity derived from stimulated values measured during oGTT did not differ (Table 2).

Concerning other hormones, normoglycemic women carrying A-allele had significantly higher level of leptin compared to women with GG genotype ($p = 0.036$) and higher level of growth hormone ($p = 0.002$) with trend to higher IGF1. They had also lower level of free triiodothyronine ($p = 0.002$), but similar TSH and free T4 levels (Table 3).

After exclusion of women using hormonal contraception, the A-allele carriers had slightly lower

Table 2. Associations of rs12970134 with glucose metabolism in normoglycemic women.

Parameters	GG (n=468)	AA+AG (n=382)	p
Glycemia (mmol/l)	4.7(4.6;4.8)	4.6(4.6;4.7)	0.001
C-peptide (nmol/l)	0.63(0.6;0.66)	0.59(0.56;0.61)	0.04
Insulin (mIU/l)	6.6(6.2;7.0)	5.8(5.5;6.2)	0.005
HbA1c (%)	4.2(4.1;4.4)	4.1(4.0;4.2)	0.04
Glycemia/Insulin (mmol/mIU)	0.71(0.67;0.77)	0.78(0.74;0.86)	0.04
AUC glycemia (mmol/l*180min)	971(948;1002)	972(952;1003)	0.67
AUC C-peptide (nmol/l*180min)	364(342;387)	363(345;374)	0.86
AUC insulin (mIU/l*180min)	4972(4600;5434)	4836(4539;5241)	0.95
AUC glycemia/insuline (mmol/mIU)	0.19(0.17;0.21)	0.20(0.18;0.21)	0.82
HOMA-IR (mIU*mmol/l ²)	1.37(1.29;1.48)	1.18(1.1;1.28)	0.002
HOMA-F (mIU/mmol)	112(105;122)	115(106;122)	0.98
Insulinogenic index (mIU/mmol)	14.6(13.4;15.9)	14.0(12.8;15.1)	0.14
Matsuda index	142(134;154)	148(136;161)	0.51
Cederholm index	73.6(70.4;77.3)	73.7(68.9;76.3)	0.47
Quicki index	0.67(0.65;0.68)	0.70(0.68;0.72)	0.002

Levels are presented as a median (95 % LCL; 95 % UCL); Mann-Whitney test.

Table 3. Associations of rs12970134 with lipid spectrum and other hormonal parameters in normoglycemic women.

Parameters	GG (n=468)	AA+AG (n=382)	p
<i>Triacylglycerols (mmol/l)</i>	0.88(0.81;0.94)	0.82(0.78;0.89)	0.78
<i>LDL-cholesterol (mmol/l)</i>	2.56(2.46;2.67)	2.41(2.34;2.59)	0.04
<i>HDL-cholesterol (mmol/l)</i>	1.49(1.44;1.54)	1.47(1.42;1.52)	0.68
<i>Total cholesterol (mmol/l)</i>	4.5(4.4;4.6)	4.4(4.3;4.5)	0.14
<i>Glucagon (pmol/l)</i>	36.1(34.5;37.5)	35.5(33.9;37.1)	0.42
<i>Leptin (mg/nl)</i>	7.5(6.6;9.4)	9.5(8.1;10.7)	0.04
<i>Growth hormone (mIU/l)</i>	1.8(1.1;2.6)	3.8(2.5;5.7)	0.002
<i>IGF-I (ng/nl)</i>	247(227;273)	267(253;285)	0.09
<i>fT4 (pmol/l)</i>	15.6(15.2;15.9)	15.6(15.3;15.9)	0.74
<i>fT3 (pmol/l)</i>	5.2(5.1;5.3)	4.9(4.8;5.1)	0.002
<i>TSH (mIU/l)</i>	2.12(2.01;2.25)	2.28(2.09;2.45)	0.24
Women not using contraceptives	GG (n=388)	AA+AG (n=288)	p
<i>Cortisol (nmol/l)</i>	476(454;491)	442.5(423;464)	0.31
<i>SHBG (nmol/l)</i>	37.2(34.6;39.1)	43.1(39.5;47.8)	0.002
<i>Testosterone (nmol/l)</i>	2.6(2.5;2.8)	2.1(1.9;2.5)	0.04
<i>Androstenedione (nmol/l)</i>	6.8(6.4;7.2)	6.2(5.8;6.6)	0.03

Levels are presented as a median (95 % LCL; 95 % UCL); Mann-Whitney test.

testosterone ($p=0.04$) and androstenedione ($p=0.03$) levels with significantly increased SHBG ($p=0.002$) in comparison to GG homozygotes (Table 3).

No significant differences in biochemical parameters between the genotypes were observed in women diagnosed with PCOS, in women with positive history of GDM, and in T2D women.

Discussion

MC4R deficiency is the most common monogenic form of human obesity which phenotype defined by I. S. Farooqi includes: early-onset obesity, increased body fat and fat-free mass, increased linear growth, preserved reproductive function, increased mineral bone density as well as hyperphagia and hyperinsulinemia which declined with age (Farooqi *et al.* 2000, 2003).

Later genome-wide association studies revealed that also common allelic variants in *MC4R* locus are associated with obesity-related phenotypes (Loos *et al.* 2008, Chambers *et al.* 2008) and their impact on anthropometric and metabolic functions had been studied.

Although most studies describe the association of the rs12970134 (A-allele) with increased risk of

obesity and obesity-related traits (Loos *et al.* 2008, Chambers *et al.* 2008, Thorliefsson *et al.* 2009, Kring *et al.* 2010, Cha *et al.* 2009), there are several studies which did not confirm such association (Sherag *et al.* 2010, Xi *et al.* 2012a, Bazzi *et al.* 2014, Albuquerque *et al.* 2014, Fernandez *et al.* 2015). In our cohort of 850 normoglycemic adult women with BMI ranging from 16 to 49.3 kg/m², no association of the minor A-allele (in dominant model) either with BMI, or with height, abdominal, waist and gluteal circumferences, WHR, subcutaneous fat and muscle mass was found. However, the higher maximum and minimum weight achieved in the adulthood was detected in women carrying the minor A-allele. Moreover, the influence of A-allele on obesity related traits was apparent in women with polycystic ovary syndrome and a trend towards higher BAI in A-allele carriers was observed also in the group of T2D women. The relation of rs12970134 and other SNPs near *MC4R* to T2D and PCOS was documented, but the potential association of these SNPs with T2D and PCOS was abolished after adjustment for BMI, indicating that a diabetogenic effect might be mediated *via* an increase in BMI (Zobel *et al.* 2009, Ewens *et al.* 2011, Louwers *et al.* 2014). However, recent meta-analysis confirmed the significant association of the rs17782313 polymorphism

near the *MC4R* gene with type 2 diabetes risk, which was independent of BMI (Xi *et al.* 2012b).

Regarding glucose metabolism, the lower fasting glycemia accompanied with lower C-peptide and insulin levels in A-allele carriers was found among normoglycemic women. These women were more insulin sensitive (HOMA-IR, Quicki indices were lower), however, these trends were not apparent after glucose administration. HOMA-IR and Quicki indices reflect rather the basal metabolic state and the increased hepatic insulin sensitivity than the peripheral/whole body one. Possible explanation suggests better hepatic function in A-allele carriers which could be influenced by preserved liver fat accumulation that was described for SNP rs17782313 near *MC4R* (Haupt *et al.* 2009).

The minor A-allele carriership was also associated with almost twice higher levels of growth hormone in normoglycemic women. In spite of high GH levels, the achieved body height in the adulthood was the same in both genotypic groups. Also other studies describe no association of rs12970134 with achieved body height but without any comment on growth hormone levels (Zobel *et al.* 2009, Kring *et al.* 2010). The study of the somatotroph axis in obese *MC4R*-deficient patients revealed increased pulsatile and total GH secretion in these patients, suggesting a role for *MC4R* in controlling hypothalamic somatostatinergic tone (Martinelli *et al.* 2011). Our data support the involvement of *MC4R* rs12970134 in regulation of somatotroph axis.

A-allele carriership was associated with higher leptin levels in our study although the BMI, WHR and subcutaneous fat did not differ from GG genotype. It could be explained by decreased leptin binding or decreased function of variant *MC4R* and moderate leptin resistance in these women. In patients with mutations of *MC4R*, serum leptin concentrations were appropriate for fat mass (Farooqi *et al.* 2003). Cole *et al.* (2010) found modest leptin resistance in some Hispanic children with *MC4R* haploinsufficiency.

Studies in rodents suggest that leptin controls the thyroid axis (Kim *et al.* 2000). Leptin communicates nutritional status to the hypothalamic-pituitary-thyroid (HPT) axis through thyrotropin-releasing hormone (TRH). In fasting state, neuropeptide Y (NPY) and *MC4R* signaling reduce thyroid hormone levels through both central pathway and peripheral hepatic circuit. Fasting-induced suppression of the HPT axis is an adaptive response in order to decrease energy expenditure

during food deprivation (Vella *et al.* 2011). In our study, free triiodothyronine levels were also affected by the *MC4R* genotype but not TSH and fT4 ones. The A-allele carriers had lower levels of peripheral fT3. This indicates the influence of *MC4R* genotype on activity of deiodases or peripheral hepatic T4 metabolism rather than on central TSH secretion. It is of note that T3 exerts the negative feedback on hypothalamic *MC4R* expression in mice (Decherf *et al.* 2010).

Melanocortins play also a role in steroidogenesis and sex function (Gantz *et al.* 2003). In our study, cortisol levels did not depend on the genotype, on the other hand, women with the minor A-allele who are not using contraceptives showed lower androgene levels such as testosterone and androstenedione compared to their GG counterparts. There are not many studies reporting influence of *MC4R* variability on steroid levels. *MC4R* deficiency due to mutated *MC4R* was associated with normal 24-h urine free cortisol, gonadal secretion, concentrations of sex steroids, and secondary sexual characteristics were appropriate for age of the affected children (Farooqi *et al.* 2003) contrary to patients with *LEPR* mutations, who manifest delayed puberty due to hypogonadotropic hypogonadism (Farooqi *et al.* 2009).

This study is a contribution to the knowledge on functional impact of variant rs12970134, which is located near *MC4R* gene, on anthropometric, metabolic and hormonal parameters. Many associations of rs12970134 found in our study were not described previously but they are in a good accordance with known functional consequences of melanocortin system and the features of hypothalamic obesity (Hochberg and Hochberg 2010).

Conflict of Interest

There is no conflict of interest.

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Abbreviations

AUC, area under the curve; BAI, body adiposity index; BMI, body mass index; fT3, free triiodothyronine; fT4, free thyroxine; GDM, gestational diabetes mellitus; HbA1c, glycated hemoglobine A1c; HOMA-IR, homeostatic model assessment – insulin resistance; HOMA-F, homeostatic model assessment – beta-cell function; IGF-1, insulin-like growth factor-1; *MC4R*,

melanocortin-4 receptor; oGTT, oral glucose tolerance test; PCOS, polycystic ovary syndrome; SHBG, sex-hormone binding globuline; SNP, single nucleotide polymorphism; T2D, type 2 diabetes; TSH, thyroid-stimulating hormone; WHR, waist to hip ratio.

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