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AVALIAÇÃO DE POLIMORFISMOS EM GENES RELACIONADOS À OBESIDADE E DIABETES EM MULHERES COM A SÍNDROME DOS OVÁRIOS POLICÍSTICOS E ASSOCIAÇÃO COM VARIÁVEIS METABÓLICAS E HORMONÀIS

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Esta Tese de Doutorado segue o formato proposto pelo Programa de Pós-Graduação em Ciências Médicas: Endocrinologia: Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, sendo apresentada na forma de manuscritos sobre o tema da Tese:

- **Artigo Original:** Polymorphisms of *TCF7L2* gene in South Brazilian women with polycystic ovary syndrome: a cross-sectional study; **Publicado** no European Journal of Endocrinology. 2013 Oct 1;169 (5):569-76

- **Artigo Original:** Association between the rs7903146 polymorphism of transcription factor 7-like 2 gene and polycystic ovary syndrome: a systematic review and meta-analysis. **Artigo Submetido.**

- **Artigo Original:** *FTO* gene variants are not associated with women with Polycystic ovary syndrome from Southern Brazil. **Artigo Submetido**

Além dos artigos já citados, ao longo do período do doutorado foram desenvolvidos os seguintes manuscritos relacionados com polimorfismos em genes ligados ao metabolismo:

- Fat mass and obesity-associated gene polymorphisms do not affect metabolic response to hormone therapy in healthy postmenopausal women. Ramos RB, Casanova GK, Spritzer PM. Eur J Obstet Gynecol Reprod Biol. 2012 Dec;165(2):302-6

- Haplotype TGTG from SNP 45T/G and 276G/T of the adiponectin gene contributes to risk of polycystic ovary syndrome. Radavelli-Bagatini S, de Oliveira IO, Ramos RB, Santos BR, Wagner MS, Lecke SB, Gigante DP, Horta BL, Spritzer PM. J Endocrinol Invest. 2013 Jul-Aug;36(7):497-502

LISTA DE ABREVIATURAS E SIGLAS

BMI/IMC – Body mass index; Índice de massa corporal

DBP - Diastolic blood pressure

DNA - Ácido desoxirribonucleico

FEM - Fixed effect model

FTO - Fat mass and obesity associated gene

HOMA-IR - Homeostasis model assessment index, estimate insulin resistance

HWE - Hardy-Weinberg equilibrium

IR/RI - Insulin Resistance; Resistência Insulínica

LAP – Lipid accumulation product

MetS - Metabolic syndrome

NOS - Newcastle-Ottawa Scale

PCOS - Polycystic ovary syndrome; Síndrome dos ovários policísticos

PCR - Polymerase chain reaction; Reação em cadeia da polimerase

REM - Random effect model

SBP - Systolic blood pressure

SHBG - Sex hormone binding globulin

SNPs - Single Nucleotide Polymorphisms

T2DM - Type 2 Diabetes; Diabetes Mellitus tipo 2

TCF7L2 - Transcription factor 7-like 2

TT - Total testosterone

WC - Waist Circumference

SUMÁRIO

RESUMO

A síndrome dos ovários policísticos (PCOS) representa uma das endocrinopatias mais frequentes em mulheres em idade reprodutiva, cujas principais características clínicas são anovulação crônica e manifestações de hiperandrogenismo. Em conjunto com os distúrbios reprodutivos, as pacientes com PCOS apresentam, frequentemente, obesidade e resistência insulínica (RI). Além disso, mulheres com PCOS apresentam maior risco para diabetes tipo 2, dislipidemia e hipertensão arterial e a presença da obesidade pode exacerbar os distúrbios metabólicos associados com a síndrome.

A patogênese da PCOS está ligada a maior susceptibilidade ambiental bem como fatores genéticos e esses fatores podem influenciar a apresentação clínica da doença. Variantes genéticas, como polimorfismos de troca de um único nucleotídeo (SNP) vem sendo associadas com alterações metabólicas e clínicas. SNPs no gene *TCF7L2* já foram descritos associados ao DM2 2 e RI. Estudos até o presente momento apresentam resultados controversos em relação a variáveis metabólicas e sua associação com os SNPs deste gene em pacientes com PCOS.

Outros genes também vem sendo estudados e sabendo que a resistência à insulina e obesidade são característica frequentes de pacientes com a PCOS, o gene *FTO* surgiu como um possível locus a ser estudado, já que diversos estudos mostram uma associação com esses fatores em outras populações. Até o presente momento estudos mostram dados controversos, possivelmente associados a diferenças entre etnias. Além disso, estudos em uma população latino americana de mulheres com PCOS ainda não foram relatados na literatura.

No presente estudo, observamos que os polimorfismos do gene do TCF7L2 rs7903146 e rs11196236 bem como seus haplótipos, não estão associados com a PCOS, mas que a paciente ser portadora de pelo menos um alelo de risco mostra uma variação positiva de 5,87 cm na cintura, 10,7 mg/dl no colesterol total e 10,3mg/dL no LDL-c. Além disso, para verificar a associação do polimorfismo rs7903146 com PCOS realizamos um meta análise, incluindo 1892 mulheres com PCOS e 2695 controles. Os

resultados sugerem que o polimorfismo no gene do *TCF7L2* não está associado com o risco aumentando de desenvolver PCOS em difefentes etnias (Asiáticas e não Asiáticas).

No que se refere ao gene do *FTO*, os polimorfismos estudados também não foram associados com PCOS, mas os resultados mostram um aumento nos níveis de glicose nas pacientes que possuíam pelo menos um alelo de risco tanto para o polimorfismo rs9939609 quanto para o rs8050136.

Estes resultados em conjunto sugerem que a PCOS por ser uma doença multifatorial e multigênica é difícil encontrar um único SNP responsável pelo fenótipo completo da PCOS, mas os estudos de associação em diferentes genes podem contribuir com o melhor entendimento dos diferentes fenótipos, principalmente nas características metabólicas destas pacientes.

ABSTRACT

The polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders in reproductive age women, whose main clinical features are chronic anovulation and hyperandrogenism. Together with reproductive disorders, patients with PCOS frequently have obesity and insulin resistance (IR). In addition, women with PCOS have a higher risk for type 2 diabetes, dyslipidemia and hypertension and the presence of obesity may exacerbate the metabolic disturbances associated to the syndrome.

The pathogenesis of PCOS is linked to greater environmental susceptibility and genetic factors and these aspects may influence the clinical presentation of the disease. Genetic variants as single nucleotide polymorphisms (SNPs) have been associated to metabolic and clinical changes. SNPs in the TCF7L2 gene have been described in association with DM2 2 and IR. Studies to date are controversial in relation to metabolic variables and their association with the SNPs of this gene in patients with PCOS.

Other genes have also been studied and knowing that insulin resistance and obesity are common characteristic in patients with PCOS, the FTO gene has emerged as a possible locus to be studied. Several studies show an association with these factors in other populations. So far studies show controversial data, possibly associated to differences among ethnic groups. In addition, studies in a Latin American women population with PCOS have not been reported in the literature.

We have found out that the gene TCF7L2, polymorphisms rs7903146 and rs11196236 and their haplotypes show no differences between genotypes and haplotypes for clinical and metabolic variables. However, for each T (rs7903146) and T (rs11196236) allele added to the haplotypes, a variation of 5.87 cm in waist (P trend=0.01), 10.7 mg/dl in total cholesterol (P trend=0.03), and 10.3 mg/dl in LDL-C (P trend=0.01) was recorded. Also, to verify the association of rs7903146 polymorphism with PCOS we conducted a meta-analysis including 1892 women with PCOS and 2695 controls. The results suggest that polymorphism in the gene TCF7L2 is not associated to

the increased risk of developing PCOS in different ethnicities (Asian and non-Asian).

As regards the FTO gene, the studied polymorphisms were not associated to PCOS, but the results show an increase in glucose levels in patients who had at least one risk allele for the polymorphism rs8050136 and rs9939609.

These results together, suggest that PCOS being a multifactorial and multigenic disease is difficult to find a single SNP responsible for the complete phenotype of PCOS, but association studies in different genes can contribute to a better understanding of the different phenotypes, especially in metabolic characteristics of these patients.

INTRODUÇÃO

Síndrome dos ovários policísticos (PCOS)

PCOS é uma doença de apresentação heterogênea cujas principais características clínicas são anovulação crônica e manifestações de hiperandrogenismo. A prevalência da PCOS pode variar de acordo com o critério de diagnóstico utilizado, estima-se que varia de 9% pelos critérios do NIH, podendo chegar a 18% se forem observados os critérios de Rotterdam (Ehrmann, Barnes et al. 1995, Knochenhauer, Key et al. 1998, Asuncion, Calvo et al. 2000), em mulheres em idade reprodutiva. Estes dados evidenciam que a PCOS apresenta grande relevância clínica, pois sua prevalência é semelhante a de doenças de grande impacto na medicina como, por exemplo, a hipertensão arterial sistêmica (HAS). Além dos distúrbios reprodutivos, as pacientes com PCOS apresentam, frequentemente, alterações metabólicas que incluem resistência insulínica, dislipidemia e maiores índices de obesidade. Em conjunto com a obesidade, outros fatores relacionados também estão associados à doença, sendo que estudos recentes demonstram que 25 a 35% das mulheres obesas com PCOS são diagnosticadas como portadoras de tolerância reduzida à glicose ou diabéticas ao redor dos 30 anos de idade (Wild, Carmina et al. 2010) e, no geral, pacientes com esta síndrome apresentam 3 a 7 vezes mais risco de desenvolver diabete mellitus tipo 2 (DM2) em comparação com a população geral (Legro, Kunselman et al. 1999). Este conjunto de fatores destaca a importância de se buscar marcadores genéticos de predisposição à doença que possam, no futuro, contribuir para a avaliação de riscos nesta população e sua prevenção.

Evidências atuais indicam que a PCOS é uma doença multifatorial e poligênica e que sua patogênese e apresentação clinica são influenciadas tanto por fatores ambientais quanto por uma maior susceptibilidade genética (Jones et al, 2012). O papel no desenvolvimento de PCOS de diversos genes envolvidos na ação e secreção de insulina, metabolismo da glicose, controle energético e características metabólicas não são totalmente conhecidos. (Biyasheva et al 2009; Ehrmann et al 2002; Radavelli-Bagatini et al 2013;

Wojciechowski et al 2012 ; Yalamanchi et al 2012) e por isso a importância de mais estudos nessa área.

***"Transcription factor 7-like 2"* (*TCF7L2*)**

Dentre os genes já estudados, alguns podem estar envolvidos com maior risco de obesidade, resistência insulínica e anormalidades metabólicas. Um destes é o gene *"Transcription factor 7-like 2"* (*TCF7L2*), localizado na região cromossômica 10q25.2. Associações entre dois polimorfismos de troca de única base (SNPs), rs7903146 e rs11196236 do gene do *TCF7L2* e DM2 foram replicados em diversos estudos, e são os polimorfismos que apresentam fortes evidências de associação com DM2 em diferentes etnias (Grant, Thorleifsson et al. 2006, Cauchi, Vaxillaire et al. 2007, Ereqat, Nasereddin et al. 2010, Barra, Dutra et al. 2012). No entanto, a associação de variantes do gene *TCF7L2* com outras alterações metabólicas são ainda controversas. Alguns estudos mostraram associação entre os polimorfismos rs7903146 e rs11196236 com variáveis ligadas a obesidade e resistência periférica a insulina (Tan, Scherag et al. 2010, Vcelak, Vejrazkova et al. 2012, Yalamanchi, Sam et al. 2012, Wang, Hu et al. 2013). Em indianos o alelo de risco do SNP rs7903146 foi associado com um aumento do colesterol total e LDL-c (Sanghera, Nath et al. 2008), enquanto que em mexicanos e finlandeses foi associado a maiores níveis de triglicerídeos (Huertas-Vazquez, Plaisier et al. 2008, Warodomwichit, Arnett et al. 2009).

Estudos prévios em PCOS não mostram resultados conclusivos. Barber et al estudando 369 mulheres com PCOS e 2574 controles do Reino Unido não observou associação do polimorfismo rs7903146 com PCOS (Barber, Bennett et al. 2007). O mesmo polimorfismo não mostrou associação em asiáticos (Xu, Che et al. 2010, Kim, Choi et al. 2012). Em uma população grega, um estudo observou uma associação entre o alelo T do SNP rs7903146 e PCOS (Christopoulos et al 2008). Em um grande estudo com 624 PCOS e 553 controles de origem Europeia não foi observado associação entre PCOS e o SNP rs7903146, contudo, foi reportado em uma região diferente do gene o polimorfismo rs11196236 e

este foi associado com variáveis metabólicas e PCOS (Biyasheva, Legro et al. 2009).

TCF7L2 é um fator de transcrição na via de sinalização Wnt, que é crítico para a embriogênese, proliferação, diferenciação e apoptose celular, bem como na manutenção da homeostase tecidual e provavelmente processos metabólicos (Smith et al., 2007). Alterações na via de sinalização Wnt estão associadas com muitas doenças que vão de câncer às doenças degenerativas, como a doença de Alzheimer (Inestrosa etl al., 2014). A sinalização Wnt foi encontrada ativada em pacientes com DM2 em comparação com normoglicêmicos. Em modelos de ratos não diabéticos, uma dieta com altos níveis de gordura induz a ativação da sinalização Wnt (Lee et al., 2008).

O mecanismo de ação que liga o gene do *TCF7L2* com obesidade e distúrbios metabólicos não está totalmente compreendido. Estudos sugerem que uma deficiência na secreção de insulina possa ser considerada como possível mecanismo responsável (Zegginie et al., 2007; Groves et al., 2006; Freathy et al., 2007). Pacientes com PCOS, em sua maioria, apresentam resistência a insulina. No entanto, além dessa alteração, evidências sugerem que concomitantemente existe um distúrbio de função de célula beta (Holte J et al., 1994), endossando a candidatura biológica de *TCF7L2* em relação a susceptibilidade à PCOS.

***Fat mass and obesity associated gene"* (*FTO*)**

Em 2007, um estudo reportou a descoberta do *"fat mass and obesity associated gene"* (*FTO*), primeiro gene associado com susceptibilidade a obesidade identificado através de "*genome wide-scan"* (Frayling, Timpson et al. 2007). Posteriormente, outros estudos associaram as variantes genéticas ligadas a este gene com obesidade/IMC (Cecil, Tavendale et al. 2008, Fang, Li et al. 2011, Webster, Warrington et al. 2011, Xi, Shen et al. 2011), DM2 (Legry, Cottel et al. 2009, Rees, Islam et al. 2011), síndrome metabólica (Frayling, Timpson et al. 2007); (Al-Attar, Pollex et al. 2008, Sjogren, Lyssenko et al. 2008), aumento do risco cardiovascular (Fisher, Schulze et al. 2009, Lappalainen, Kolehmainen et al. 2010, Zimmermann, Skogstrand et al.

2011) e maiores níveis de insulina (Karasawa, Daimon et al. 2010). O fato deste gene estar associado com alterações metabólicas frequentemente observadas em pacientes com PCOS, levou à hipótese que variantes genéticas do gene *FTO* poderiam estar diretamente associadas à suscetibilidade ao PCOS.

O gene do *FTO* está localizado na região cromossômica 16q12.2 e é formado por 9 éxons e 8 introns. Nele já foram identificados mais de 2.348 SNPs. Destes SNPs, 92 têm conhecida importância científica, dos quais 26 estão relacionados com IMC (Rampersaud, Mitchell et al. 2008). Entre todos estes polimorfismos podemos destacar o SNP rs9939609 (troca de uma timina (T) por uma adenina (A)) e rs8050136 (troca de uma A por uma citosina (C)) ambos localizados no intron 1 do gene e separados por 4251 pares de base. Estes polimorfismos já foram descritos em um bloco com um forte desequilíbrio de ligação (Hotta, Nakata et al. 2008).

O gene *FTO* em humanos é expresso principalmente no cérebro, mais especificamente *na* região do núcleo arqueado do hipotálamo, que é o responsável pelo controle energético e também na glândula pituitária e adrenais, sugerindo que esse gene tenha um papel importante no eixo hipotálamo-hipófise-adrenal (Dina, Meyre et al. 2007, Frayling, Timpson et al. 2007, Gerken, Girard et al. 2007, Stratigopoulos, Padilla et al. 2008). Além disso, estudos demonstraram expressão do gene também no tecido adiposo, pâncreas, fígado, musculatura esquelética estriada, cardíaca e rins (Gerken, Girard et al. 2007, Stratigopoulos, Padilla et al. 2008). Essa expressão em diversos tecidos indica que o *FTO* pode ter uma função importante, possivelmente na regulação do peso corporal através da homeostase de energia controlando o gasto energético (Frayling, Timpson et al. 2007, Sanchez-Pulido and Andrade-Navarro 2007, Fischer, Koch et al. 2009). Entretanto, seu real papel e mecanismos de ação ainda não foram totalmente esclarecidos (Tung and Yeo 2011).

Estudos realizados até o presente momento, sugerem que polimorfismos no gene do *FTO* possam estar associados com variáveis antropométricas. Um estudo na população polonesa observou um grande impacto do SNP rs9939609 no peso corporal de mulheres com PCOS (Kowalska, Malecki et al. 2009), Neste estudo uma diferença de 10 kg foi

encontrada entre o genótipo em homozigose AA quando comparado com o genótipo em homozigose TT. Outros quatro estudos também reportaram um efeito maior do que o esperado do gene do *FTO* no peso em diferentes coortes de pacientes com PCOS (Wehr, Schweighofer et al. 2009; Barber, Bennett et al. 2008; Ewens KG et al., 2001; Tan, Scherag et al., 2010). Tan e colaboradores (2010) também mostraram que ser portadora do genótipo polimórfico para o SNP rs9939609 está associado com variáveis relacionadas a resistência à insulina (insulina e HOMA-IR). Além desses resultados o estudo mostrou que a presença do alelo polimórfico (A) também foi maior em pacientes com a PCOS sem a aparência policística do ovário quando comparado com pacientes que apresentavam somente aparência policística do ovário sem a síndrome (Tan, Scherag et al. 2010).

Dois diferentes estudos, um numa população do Reino Unido e outro em chinesas, observaram uma associação entre o alelo A do SNP rs9939609 e o diagnóstico de PCOS e esta associação se mostrou mais forte em pacientes obesas, sugerindo que possivelmente essa maior susceptibilidade é mediada pelo efeito do IMC (Barber, Bennett et al. 2008; Yan, Hong et al. 2009). No entanto, outros estudos não confirmaram essa associação (Kowaslka, Malecki et al., 2009; Wehr, Schweighofer et al., 2009).

No que se refere aos androgênios, resultados conflitantes são observados entre estudos: enquanto dois trabalhos não observaram associação entre o polimorfismo e níveis de androgênios em mulheres com PCOS, diagnosticadas pelo consenso de Rotterdam (Barber, Bennett et al. 2008, Kowalska, Malecki et al. 2009), Wehr et al. descreveu uma associação entre o alelo A do SNP rs9939609 e níveis de testosterona livre circulante. (Wehr, Schweighofer et al. 2009).

Com base nestes conhecimentos, o objetivo deste estudo foi avaliar a associação de genes ligados a obesidade e DM2 (*FTO* e *TCF7L2*) em pacientes com PCOS e controles saudáveis, bem como sua associação com variáveis clinicas e metabólicas.

Parte 1

Polymorphisms of *TCF7L2* gene in South Brazilian women with polycystic ovary syndrome: a cross-sectional study

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Polymorphisms of TCF7L2 gene in South Brazilian women with polycystic ovary syndrome: a cross-sectional study

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Abstract

Objective: To assess whether TCF7L2 single nucleotide polymorphisms rs7903146 C/T and rs11196236 C/T are associated with polycystic ovary syndrome (PCOS) in South Brazilian women. *Design:* Cross-sectional study.

Methods: 200 PCOS patients and 102 non-hirsute, ovulatory controls were genotyped by real-time polymerase-chain reaction. Haplotypes were constructed from the combination of both polymorphisms. Frequencies were inferred using the PHASE 2.1.1 software.

Results and Conclusions: The distribution of rs7903146 (PCOS, 54.4% CC; 28.5% CT; 17.1% TT; controls, 51.0% CC; 37.0% CT; 12.0% TT) and rs11196236 (PCOS, 4.3% CC; 33.5% CT; 62.2% TT; controls, 3.2% CC; 35.5% CT; 61.3% TT) was similar between the groups. rs7903146 and rs11196236 were not in linkage disequilibrium (|D´|=0.34; r2=0.07). PCOS participants were younger, with higher age-adjusted BMI, waist circumference, blood pressure, triglycerides, insulin, HOMA-IR and total testosterone, and lower HDL-c and SHBG vs. controls. In PCOS, the TT genotype in rs7903146 was associated with higher BMI and waist circumference vs. CC and CT; the TT genotype in rs11196236 was associated with higher waist circumference, total cholesterol, and LDL-c vs. CC and CT. Haplotypes TTCT and TTTT were associated with higher waist circumference and LDL-c vs. CCCC, CCCT and CCTC. For each TT allele added to the haplotypes, a variation of 5.87 cm in waist (P trend=0.01), 10.7 mg/dL in total cholesterol (P trend=0.03), and 10.3 mg/dL in LDL-c (P trend=0.01) was recorded. TCF7L2 variants are probably not implicated in PCOS development, but may be associated with metabolic traits in South Brazilian women with PCOS.

Introduction

Polycystic ovary syndrome (PCOS) is a complex endocrine disease characterized by hyperandrogenism and chronic anovulation. It is associated with metabolic disturbances such as central obesity, insulin resistance, dyslipidemia and increased risk of diabetes and hypertension (1-6).

Current evidence indicates that PCOS is a multifactorial polygenic disorder, whose pathogenesis and clinical presentation are influenced by both genetic susceptibility and environmental exposure (5, 7). To date, a number of genes have been reported to be associated with PCOS. Most of them are presumed to be relevant to the pathogenesis of PCOS, such as the genes encoding steroid biosynthesis enzymes and androgen and insulin receptors. However, a variant contributing substantially to the development of PCOS has not been identified (8, 9).

Recently, common variants of the transcription factor 7-like 2 (TCF7L2) gene on chromosome 10q25.2 have been found to contribute to the risk of type 2 diabetes (T2DM) in various ethnic groups (10-13). Association studies on the single nucleotide polymorphisms (SNPs) of the TCF7L2 gene in PCOS women have produced controversial data, with some studies showing association between SNPs rs7903146 and rs11196236 with obesity-related traits (14) and peripheral insulin resistance (15), and others reporting no such association (16, 17).

Therefore, the aim of our study was to assess whether TCF7L2 SNPs rs7903146 C/T and rs11196236 C/T or their haplotypes are associated with PCOS and to determine a possible impact of these polymorphisms on anthropometric and metabolic variables in PCOS women from southern Brazil.

Materials and Methods

Patients

We studied 302 women, including 200 PCOS patients and 102 non-hirsute women with regular ovulatory cycles (luteal phase progesterone >3.8ng/ml). All participants were of reproductive age, consulting at a university hospital in Brazil or recruited by advertisement in local media between 2009 and 2012. PCOS was diagnosed according to Rotterdam criteria, in the presence of two out of three signs: 1) chronic anovulation, 2) clinical and/or biochemical hyperandrogenism and 3) polycystic ovaries (PCO). Diagnosis of PCOS also relied on exclusion of other hyperandrogenic disorders (18). None of the PCOS or control participants had received any drugs known to interfere with hormonal levels (such as OC pills, antiandrogens, metformin, fibrates, statins) for at least 3 months before the study. Exclusion criteria were: pregnancy, hepatic or renal diseases. Approval for this study was obtained from the Institutional Review Board and the local Ethics Committee, functioning according to the 3rd edition of the Guidelines on the Practice of Ethical Committees in Medical Research issued by the Royal College of Physicians of London, and written informed consent was obtained from every subject.

Study Protocol

Participants were evaluated according to a standard protocol, as previously reported (19, 20). Anthropometric measures included weight, height, and waist circumference (waist measured at the mid- point between the lower rib margin and the iliac crest) (21). Body mass index (BMI) was then calculated (current weight in kilograms divided by square of height in meters). Blood pressure was measured after a 10-minute rest, in the sitting position, with feet on the floor and the arm supported at heart level. Two measurements were performed 10 minutes apart using automatic

blood pressure monitor HEM-742INT OMRON (Rio de Janeiro, Brazil) with correct cuff size for the arm diameter. Hypertension was defined as systolic blood pressure ≥ 140 mmHg, diastolic blood pressure ≥ 90 mmHg, or current use of antihypertensive drugs (22). Hormonal and metabolic assessments were made between days 2 and 10 of the menstrual cycle, or on any day if the patient was amenorrheic. All samples were obtained between 8 and 10 AM. Blood samples were drawn after a 12-hour overnight fast for determination of plasma cholesterol, HDL-cholesterol, triglycerides, total testosterone (TT) and sex hormone binding globulin (SHBG). Glucose was measured before and 2 hours after the ingestion of a 75-g oral glucose load.

Assays

Total cholesterol, HDL-c, triglycerides, and glucose were determined by enzymatic colorimetric methods (Bayer 1650 Advia System). LDL cholesterol was estimated indirectly with the Friedewald formula (23). The lipid accumulation product (LAP) index was calculated using the formula [waist (cm) – 58] x triglyceride concentration (mmol/L) (24). Homeostasis model assessment index to estimate insulin resistance (HOMA-IR) was calculated by multiplying insulin (mIU/mL) by glucose (mmol/L) and dividing this product by 22.5 (25). Frequency of the metabolic syndrome were defined in accordance with the Joint Scientific Statement (26).

TT levels were measured by chemiluminescence (Siemens Advia Centaur XP), with a sensitivity of 0.10 ng/mL and intra-assay and interassay coefficient of variation (CV) of 3.3% and 7.5%, respectively. SHBG was measured by chemiluminescence (Immulite 2000 Siemens), with a sensitivity of 0.02 nmol/L and

intra-assay and interassay CV of 5.3% and 6.6%, respectively. Plasma insulin levels were measured by electrochemiluminescence (Siemens Advia Centaur XP), with a sensitivity of 0.50 U/mL and intra-assay and interassay CV of 2.8% and 2.1%, respectively.

Genotyping

In addition to serum samples, whole blood samples were collected from all participants. Genomic DNA was extracted from peripheral leukocytes using the technique described by Miller et al. (27). DNA samples were diluted to 2 ng/mL and genotyped for SNP 7903146 C>T and rs11196236 C>T of the TCF7L2 gene by real-time polymerase chain reaction (7500 Fast; Applied Biosystems, California, USA), with allelic discrimination assays (Taqman MGB Probes) following the manufacturer's instructions (Applied Biosystems, California, USA). Reaction conditions for SNP rs7903146 were: 10 min at 95°C after 60 cycles of denaturation at 95°C (15 s) and annealing at 62 °C (1 min). Reaction conditions for rs11196236 were: 10 min at 95°C after 50 denaturation cycles at 95°C (15 s) and annealing at 62°C (1 min). Endpoint fluorescent readings were performed by 7500 Fast System Sequence Detection Software version 1.4. In this sample 10% were made in duplicate.

After frequencies analyses, a dominant model was assumed and PCOS carriers of allele C were analyzed together in both polymorphisms. Therefore, Lewontin's D' (|D'|) r2 was calculated for each pair of genetic markers for estimating linkage disequilibrium (28, 29). Haplotypes were constructed from the combination of the two TCF7L2 polymorphisms (rs7903146 and rs11196236), and their frequencies were inferred using the PHASE 2.1.1 software (30). The first letter in

each haplotype refers to the rs7903146 polymorphism, and the second to the rs11196236 polymorphism.

Statistical analysis

Sample size estimation was based on the study by Xu et al (33), which studied an association between SNP rs7903146 of the TCF7L2 gene and anthropometric characteristics in PCOS women. Therefore, considering a difference of 1.5 in BMI between the genotypes, an alpha of 5%, and a beta of 80%, the sample size was estimated as 227 in PCOS group and 69 in control group. A subsequent interim analysis showed that a difference of 1.5 in BMI was actually obtained with the sample of 200 PCOS enrolled in the study.

Results are presented as mean ± SD, or median and interquartile range. Logarithmic transformation was used to normalize distribution of non-Gaussian variables in order to allow comparisons between the groups with independent t-test and one-way ANOVA. Bonferroni *post-hoc* test was applied to determine which groups differed significantly. A test for linear trend was used to test co-dominant effects of genotypes on dependent variables. All analyses were performed using the Statistical Package for the Social Sciences (SPSS version 20, Chicago, IL, USA). Data were considered to be significant at $P<0.05$.

Results

Participants were mostly Caucasian (94.2%), with the remaining participants having mixed (African and European) ancestry. Mean age was 23.7 ± 7.1 years. In the PCOS group, 25.5% patients had metabolic syndrome and 25.3% had hypertension. In the control group, 2.3% participants had metabolic syndrome and

6.9% had hypertension.

Table 1 summarizes the clinical, metabolic, and hormonal profile of PCOS and control participants. Total cholesterol, LDL-c and fasting glucose were similar between the groups. Women with PCOS were significantly younger than controls. As expected, women with PCOS also had significantly higher BMI, systolic blood pressure (SBP), diastolic blood pressure (DBP), waist circumference, serum triglycerides, glucose at 120 min, fasting insulin, HOMA-IR, LAP, and TT, and lower HDL-c and SHBG in comparison to the control group. These results remained significant even when adjustment for age.

For the 200 women in the PCOS group, the frequencies of genotypes for polymorphism rs7903146 were as follows: 54.4% for CC genotype, 28.5% for heterozygous CT and 17.1% for TT genotype. These frequencies did not differ from those recorded for the control group: 51.0% for CC genotype, 37.0% for heterozygous CT, and 12.0% for homozygous TT genotype respectively (table 2). Of the attempted genotypes, 96.5% were successful for this SNP. Genotypic distributions of SNP rs11196236 in women with PCOS were: 4.3% for CC genotype, 33.5% for heterozygous CT, and 62.2% for homozygous TT genotype. Similar frequencies were observed in controls: 3.2% for CC, 35.5% for heterozygous CT, and 61.3% for homozygous TT genotype (table 2). Of the attempted genotypes, 93.0% were successful for this SNP. The genotype distributions for both SNPs were in Hardy-Weinberg equilibrium in controls.

The rs7903146 polymorphism was not in linkage disequilibrium with the rs11196236 polymorphism ($|D'| = 0.34$; $r2 = 0.07$). Ten haplotypes were inferred in this sample (Ht1: CCCC, Ht2: CCCT, Ht3: CCTC, Ht4: CTCT, Ht5: CTTC, Ht6: CTTT, Ht7: TCTC, Ht8: TCTT, HT9: TTCT, HT10: TTTT). Haplotype frequencies

were 18.5% for Ht1/Ht2/Ht3, 69.5% for Ht4/Ht5/Ht6/Ht7/Ht8, and 12.0% for Ht9/Ht10.

Subsequent analyses were made specifically with the PCOS group. Table 3 shows clinical and metabolic variables according to SNP rs7903146 genotypes. Individuals with the TT genotype had significantly higher BMI and waist circumference compared with individuals presenting the CC and the CT genotypes. There were no differences between genotypes for any of the other studied variables.

Table 4 presents clinical and metabolic variables according to SNP rs11196236 genotypes in PCOS. Individuals with the TT genotype had significantly higher waist circumference, total cholesterol and LDL-c compared with individuals presenting the CC and CT genotype. No statistical differences were observed among the different genotypes for the other studied variables.

Table 5 shows clinical and metabolic variables according to haplotypes in PCOS. Individuals with the Ht9/Ht10 haplotype had significantly higher waist circumference and LDL-c compared with Ht1/Ht2/Ht3. In addition, for each TT allele added to the haplotypes a variation of 5.87 cm in waist (p trend= 0.01), 10.7 mg/dL in total cholesterol (p trend= 0.03), and 10.3 mg/dL in LDL-c (p trend = 0.01) was recorded (Figure 1).

Discussion

In the present study, SNPs rs7903146 and rs11196236 of the TCF7L2 gene were not associated with the occurrence of PCOS in women from South Brazil. Previous genetic association studies with PCOS patients, although not entirely conclusive, have shown similar results. Barber et al., studying 369 women with PCOS and 2574 controls of UK British or Irish origin, and 540 PCOS and 1083

controls from the Northern Finland Birth Cohort of 1966 found no association of polymorphism rs7903146 with PCOS (31). That was also the case with another large study analyzing 624 PCOS and 553 control women of European ancestry, which did not find an association between rs7903146 of TCF7L2 and PCOS. However, the latter study reported that variation in a specific region of the gene, where SNP rs11196236 is located, was associated with reproductive and metabolic quantitative traits in PCOS (32). Studies focusing on SNP rs7903146 in Chinese (33) and Korean women (34) were also unable to detect an association with PCOS. The genotype distribution of the TCF7L2 variants found in our PCOS women and controls agree with these earlier findings (14, 31).

Interestingly, we found an association between the TT genotype of the rs7903146 polymorphism and obesity-related traits in patients with PCOS, while SNP rs11196236, as well as haplotypes Ht9/Ht10, was associated with waist circumference and LDL-c. While very few studies are available in PCOS populations, studies with diabetic individuals have shown analogous associations of TCF7L2 and obesity-related traits (14, 35, 36). In North India, Sanghera et al. observed increased cholesterol and LDL-c levels in risk alleles carriers of SNP rs7903146 (37); SNP rs7903146 was associated with high triglyceride levels in Mexican and Finnish populations (38, 39). Moreover, a meta-analysis indicated an association between the T allele of rs7903146 and the metabolic syndrome (40). In contrast, other studies with diabetic populations did not find an influence on body weight (37, 41, 42).

It is well established that common polymorphisms of the TCF7L2 gene are associated with T2DM (10, 41, 43, 44). Grant et al. have shown that microsatellite DG10S478 of the TCF7l2 gene increases the risk of T2D by approximately 1.45 in heterozygotes and 2.41 in homozygotes. In that study, DG10S478 was in strong

linkage disequilibrium with rs7903146 (10). The TT genotype of rs7903146 also predicted the risk of diabetes in the Diabetes Prevention Program (DPP) (36) and the Finnish Diabetes Prevention Study (45).

In this sense, studies demonstrate that variation in the TCF7L2 gene is associated with defects in insulin secretion (46-48). Greater proinsulin/insulin ratio has consistently been observed among rs7903146 T allele carriers; contrarily, associations with proinsulin level have been less consistent (42, 47, 49). In PCOS women with abnormal glucose tolerance, TCF7L2 polymorphisms were associated with defects in insulin secretion (32). Another study with a small sample of 31 PCOS women has found that SNP rs11196236 was associated with peripheral insulin (15). In contrast, similarly to other studies with PCOS populations (14, 33, 34), we did not find an association between insulin resistance and SNPs of the TCF7L2. It should be noted that differences in anthropometry, proportion of less severe phenotypes, and ethnicity among these PCOS populations could have, at least in part, masked the influence of TCF7L2 SNPs on clinical expression of insulin resistance.

The mechanism linking the TCF7L2 gene with obesity traits and metabolic disturbances in diabetes has not been established. TCF7L2 encodes a transcriptional factor that mediates downstream Wnt signaling after binding with beta-catenin. Some evidence indicates that TCF7L2 is significantly expressed in human target tissues for glucose homeostasis, including visceral and subcutaneous fat (45, 50, 51) and that Wnt signaling could be a key regulator of adipogenesis through the beta-catenin/TCF7L2 dependent pathway (52). Thus, overexpression of Wnt signaling might block adipogenesis, whereas inhibition of TCF7L2 could stimulate adipogenesis, as observed *in vitro* (53). Such observations further support multiples effects of TCF7L2 on homeostasis through modulation of adipogenesis. However, it

is unclear how these intronic variants of the TCF7L2 gene may influences phenotypes. It is likely that these intronic variants act by affecting the expression of TCF7L2 rather than altering the structure of expressed protein (54). Wnt signaling could also affect insulin secretion (55). One hypothesis suggests that genetic variation in the TCF7L2 gene might impair the expression of glucagone-like-peptide 1 (GLP-1), possibly by Wnt/TCF7L2 pathways, which influences insulin secretion from the pancreatic β cells (10, 56).

One limitation of the present study was the relatively small sample size of 300 participants. However, the effect sizes observed in our sample are similar to those reported in other PCOS populations. Our data for polymorphisms rs7903146 and rs11196236 and haplotypes of the TCF7L2 gene provide evidence of an association with some quantitative traits in women with PCOS from southern Brazil. However, further studies are needed to elucidate the mechanisms through which TCF7L2 risk genotypes and haplotypes could influence obesity related-traits and metabolic components, and to establish further consequences of the interactions between genetic and environmental factors.

In conclusion, the present results suggest that variants of the TCF7L2 gene are not implicated in the development of PCOS, but may be associated with specific metabolic aspects in women with PCOS from southern Brazil.

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Parte 2

Association between the rs7903146 polymorphism of transcription factor 7-like 2 gene and polycystic ovary syndrome: a systematic review and meta-analysis

Association between the rs7903146 Polymorphism of Transcription factor 7-like 2 gene and Polycystic Ovary Syndrome: a systematic review and meta-analysis

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Running title: *TCF7L2* gene polymorphism and PCOS: a meta-analysis

The authors declare that there are no conflicts of interest.

Abstract

Purpose: The present systematic review and meta-analysis of case-control studies examines the association between rs7903146 polymorphism of the *TCF7L2* gene and PCOS. **Methods:** A search of the literature published until August 2014 was carried out in PubMed, Cochrane Central Register of Controlled Trials, EMBASE and LILACS. There were no other limits except for the end date. We included observational studies with women of any age diagnosed with PCOS and healthy controls that analyzed polymorphism rs7903146. **Results:** We included case-control or cross-sectional studies analyzing polymorphism rs7903146 in women with PCOS and healthy controls. Eighteen studies were identified after the primary literature search and 7 articles were included in the meta-analysis. All employed Rotterdam criteria for diagnosis of PCOS. The genotypic distributions in the control groups were in agreement with Hardy-Weinberg equilibrium in all studies. The pooled population included Asian descendents (Chinese, Korean), Caucasians from southern Brazil, Tunisian and European populations (British/Irish, Northern Finns, Greeks, Czechs), including 1892 women with PCOS and 2695 controls. There were no significant associations between PCOS and *TCF7L2* rs7903146 polymorphism, irrespective of whether allele contrast, additive, dominant or recessive models of inheritance were used. Furthermore, no significant associations were found after stratification for ethnicity (Asian or non-Asian). There was no significant heterogeneity between studies and no publication bias. **Conclusions:** the present results suggest that the rs7903146 T allele is not associated with risk for PCOS in in non-Asian or Asian women. This systematic review and meta-analysis is registered in PROSPERO under number CRD42013005930.

Keywords

Meta-Analysis; Polycystic Ovary Syndrome; Review, Systematic; Transcription Factor 7-Like 2 Gene

Introduction

Polycystic ovary syndrome (PCOS) is a complex and common endocrine disease that affects women of reproductive age. The worldwide prevalence of PCOS varies between 9 and 18%, depending on diagnostic criteria (Azziz, Woods et al. 2004, March, Moore et al. 2010). The classical, and most severe, PCOS phenotype is characterized by hyperandrogenism and chronic anovulation (Azziz, Carmina et al. 2009), and is associated with metabolic disturbances such as central obesity, insulin resistance, dyslipidemia, and increased risk of diabetes and hypertension (Ehrmann 2005, Norman, Dewailly et al. 2007, Azziz, Carmina et al. 2009, Berneis, Rizzo et al. 2009, Wild, Carmina et al. 2010, Diamanti-Kandarakis, Spritzer et al. 2012). The rate of impaired glucose tolerance is 3-fold higher in PCOS women as compared to non-PCOS women of similar age (Legro, Kunselman et al. 1999), and the prevalence of undiagnosed type 2 diabetes is 7.5 to 10 fold higher than in women of similar age from the NHANES II (Ehrmann, Barnes et al. 1999, Legro, Kunselman et al. 1999).

Current evidence indicates that PCOS is a multifactorial polygenic disorder whose pathogenesis and clinical presentation are influenced by both genetic susceptibility and environmental exposure (Jones, Chua et al. 2012). The role played in the development of PCOS by the many genes involved in insulin action and secretion, glucose metabolism, energy metabolism, and metabolic traits are unknown (Ehrmann, Tang et al. 2002, Xita, Georgiou et al. 2002, Biyasheva, Legro et al. 2009, Wojciechowski, Lipowska et al. 2012, Yalamanchi, Sam et al. 2012, Radavelli-Bagatini, de Oliveira et al. 2013).

Transcription factor 7-like 2 (*TCF7L2*) gene, coded at chromosomal region 10q25.2, is involved in the Wnt signaling pathway, which plays a crucial role in cell proliferation, differentiation and apoptosis, in the maintenance of tissue homeostasis, and probably in metabolic processes (Smith 2007). Several studies have reported an association between common variants in the *TCF7L2* gene and T2D (Grant, Thorleifsson et al. 2006, Groves, Zeggini et al. 2006, Zeggini and McCarthy 2007). Also, T2D development seems to be predicted by the T risk allele of rs7903146, which is associated with increased pancreatic cell *TCF7L2* gene expression and

decreased insulin secretion (Saxena, Gianniny et al. 2006, Lyssenko, Lupi et al. 2007). The biological candidacy of *TCF7L2* with respect to the susceptibility to PCOS is further supported by the fact that GLP-1, which stimulates insulin secretion and biosynthesis and inhibits glucagon release, is a product of the proglucagon gene – coded by *TCF7L2* (Deacon 2004, Yi, Brubaker et al. 2005).

To date, two reviews have been published on this subject, the first being a meta-analysis including studies published up to November 2013 (Shen, Li et al. 2014). The second is a recently published systematic review, not followed by a meta-analysis (Lin, Yang et al. 2014).

The present systematic review and meta-analysis of case-control studies examines the association between rs7903146 polymorphism of *TCF7L2* gene and PCOS, diagnosed by the Rotterdam criteria and included studies up to August 2014.

Methods

Search strategy and study selection

A search of the literature published until August 2014 was carried out in PubMed, Cochrane Central Register of Controlled Trials, EMBASE and LILACS. There were no other limits except for the end date. This systematic review and meta-analysis is reported in accordance with current guidelines (Stroup, Berlin et al. 2000, Liberati, Altman et al. 2009) and registered in PROSPERO (http://www.crd.york.ac.uk/PROSPERO/) under number CRD42013005930.

The search strategy consisted of the following medical subject headings (MeSH): “Polycystic Ovary Syndrome” OR “Ovary Syndrome, Polycystic” OR “Syndrome, Polycystic Ovary” OR “PCOS” AND “*TCF7L2* Transcription Factor” OR “ Transcription Factor Like 2” OR “*TCF7L2*” AND “polymorphism, genetic” OR “polymorphism, single nucleotide”. We included observational studies (case-control or cross-sectional designs) with women of any age diagnosed with PCOS and healthy controls that analyzed polymorphism rs7903146. A hand search was also performed in the reference lists of full text articles. Studies were excluded from the analysis if the genotype distributions in the control group deviated from those predicted by Hardy-

Weinberg equilibrium (HWE) or if they did not have sufficient data to estimate an OR with 95% confidence interval. If data were duplicated or had reported more than once, the most complete study was chosen.

Data extraction and quality control assessment

Two investigators (R.B.R and V.C.F) independently reviewed the titles and abstracts of all articles selected in order to evaluate whether the studies were eligible for inclusion in the meta-analysis. The selected articles were read in full to confirm eligibility and to extract data. Disagreements were resolved by discussion between these two investigators. When necessary, a third reviewer (P.M.S) was consulted. Where abstracts did not provide enough information regarding the inclusion and exclusion criteria, the full text of the article was retrieved for evaluation. The information extracted from each individual study was as follows: name of first author, publication year, country, number of subjects in case and control groups, age, genotype and allele frequencies in case and control subjects and OR (95% CI).

The Newcastle-Ottawa Scale (NOS) (Wells, Shea et al. 2013) was used to assess the quality of nonrandomized studies by two independent investigators (R.B.R and V.C.F). Using this 'star system,' each study included was judged on three broad perspectives: selection of the study groups; comparability of the groups; and ascertainment of outcome of interest.

Statistical analysis

The genotype distributions of control subjects were tested for conformity with HWE using a goodness-of-fitness χ^2 test. Gene-disease associations were measured using estimation of odds ratios (OR) (95% CI) based on the following genetic inheritance models: 1) allele contrast (C vs. T); 2) additive model (TT vs. CC); 3) recessive model (CC/CT vs. TT); 4) dominant model (CC vs. CT/TT) (Minelli, Thompson et al. 2005, Zintzaras and Lau 2008).

Heterogeneity was tested using a χ^2-based Cochran's Q statistic test. Inconsistency was assessed with the I^2 metric. Heterogeneity across the eligible studies was tested using the Q-test. $p< 0.10$ was considered statistically significant. Heterogeneity was also quantified with the I^2 test, with

I^2 >50% defined as heterogeneity for the I^2 metric statistic. Where significant heterogeneity was detected (p <0.10, I^2 >50%), the DerSimonian and Laird random effect model (REM) was used to calculate OR (95% CI) for each individual study and for the pooled effect; where heterogeneity was not significant, the fixed effect model (FEM) was used for this calculation (Higgins and Thompson 2002, Higgins, Thompson et al. 2003).

Risk of publication bias was assessed using funnel plot graphics, and an asymmetric plot suggested possible publication bias. Egger's test, which calculates the funnel plot asymmetry of the ORs on a natural logarithm scale based on a linear regression, was used to assess the funnel plot asymmetry (Egger, Davey Smith et al. 1997). The significance of the intercept was determined by the t test, as proposed by Egger, with p = 0.10 considered indicative of statistically significant publication bias. All statistical analyses were performed using Stata 11.0 software (StataCorp, College Station, TX, USA).

Results

Studies selection

Figure 1 shows the strategy used to identify and select studies for inclusion in the meta-analysis. Eighteen potentially eligible studies were identified after the primary literature search. Five articles were excluded after screening of titles and abstracts, because they did not focus on the polymorphism of interest to the present study. The full text of the thirteen articles was reviewed. That resulted in the exclusion of two review articles (Lin, Yang et al. 2014, Shen, Li et al. 2014) and an additional article that lacked a control group (Tan, Scherag et al. 2010), and ten studies were included in the qualitative synthesis. Of these ten articles, three were excluded from the meta-analysis because they did not present the genotype frequencies (Biyasheva, Legro et al. 2009, Ewens, Jones et al. 2011, Yalamanchi, Sam et al. 2012). Therefore, a total of seven articles meeting eligibility criteria were included in the meta-analysis. The genotypic distributions in the control group of each study were in agreement with HWE, with χ^2 <3.84 (p>0.05).

Characteristics of the included studies

Table 1 provides the characteristics of the studies included in the meta-analysis. Two studies included Asian descendents (Chinese, Korean) (Xu, Che et al. 2010, Kim, Choi et al. 2012). One study included Caucasians from southern Brazil (Ramos, Wiltgen et al. 2013), another study included Tunisian women from North African community (Ben-Salem, Ajina et al. 2013), and the remaining three studied European populations (British/Irish, Northern Finns, Greeks, Czechs) (Barber, Bennett et al. 2007, Christopoulos, Mastorakos et al. 2008, Vcelak, Vejrazkova et al. 2012). Regarding the study of Barber et al (2007) they studied a heterogeneous group of participants, including 358 UK cases with a definitive diagnosis of PCOS according to the Rotterdam criteria, 540 women from the Northern Finland Birth cohort, presenting PCOS symptoms, but not a Rotterdam criteria-based PCOS diagnosis, and a control group that included men. Therefore, in the present study, only the 358 PCOS and 1243 control women were included in the meta-analysis.

Study size ranged from 119 to 377 patients, with a total 1892 women with PCOS and 2695 controls in the present meta-analysis of the relationship between *TCF7L2* polymorphism and susceptibility to PCOS. The seven studies that entered in the meta-analysis employed Rotterdam criteria for diagnosis of PCOS. Scores of the NOS of the included studies ranged from 5 to 7 (Table 2).

The mean age of the PCOS patients ranged from 22.8 to 32.0 years, and that of controls ranged from 25.2 to 29.9 years. Mean BMI ranged from 22.2 to 30.2 kg/m^2 in PCOS and 20.9 to 27.0 kg/m^2 in controls.

Association analysis between rs7903146 SNP and PCOS

Gene-disease associations were measured for the following genetic inheritance models: allele contrast, additive, recessive and dominant. All the genetic inheritance models were performed after stratification by ethnicity (Asian and non-Asian). In one study the frequency of TT genotype was 0, and for this reason the study was excluded from recessive and additive analyses of inheritance models (Kim, Choi et al. 2012). Figure 2 illustrates the pooled OR for the associations between *TCF7L2* polymorphism and PCOS in

different models, assuming an “A” allele contrast model, “B” additive model, “C” dominant model, “D” recessive model.

Our results revealed no significant associations between PCOS and *TCF7L2* rs7903146 polymorphism, irrespective of whether allele contrast, additive, dominant or recessive models of inheritance were used. Furthermore, no significant associations were found after stratification for ethnicity (Figure 2). Significant heterogeneity (Q test p >0.10 and I^2 <50%) between studies investigating rs7903146 was not identified regardless of inheritance model.

Publication bias

No apparent publication bias was found by either visual inspection of the funnel plot or formal statistical tests: Egger’s regression test and Begg’s rank correlation test indicated that our data are statistically robust in any inheritance model (Figure 3).

Discussion

The present meta-analysis including seven case-control studies and 1892 women with PCOS were conducted to explore the possibility that the rs7903146 polymorphism of *TCF7L2* gene was associated with PCOS. The present results showed that there seems to be no association between this polymorphism and PCOS, both for Asian and non-Asian populations.

Our study differs in crucial aspects from the other meta-analysis reporting that rs7903146, but not rs12255372 polymorphism, may contribute to susceptibility to PCOS (Shen, Li et al. 2014). One concern regarding that meta-analysis is that the authors found associations between the rs7903146 polymorphism and PCOS that were not reported by the authors in their original articles (Barber, Bennett et al. 2007, Vcelak, Vejrazkova et al. 2012). Another limiting aspect was the absence of genotype frequency for each of the studies reviewed, an aspect that prevents reproduction of the analyses performed. A third point is the inclusion of all the heterogeneous groups studied by Barber et al., including women with PCOS symptoms, but with no confirmed diagnosis and a control group that included men. In our study, only cases with confirmed diagnosis of PCOS by Rotterdam criteria and control

women were included. A systematic review conducted recently also found similar results to the present study, even if no meta-analysis was performed (Lin, Yang et al. 2014).

The rs7903146 polymorphism of the *TCF7L2* gene has been the most studied with respect to PCOS (Christopoulos, Mastorakos et al. 2008, Vcelak, Vejrazkova et al. 2012, Ramos, Wiltgen et al. 2013), but studies have been limited by their small sample size (Vcelak, Vejrazkova et al. 2012, Ramos, Wiltgen et al. 2013). In that context, meta-analyses may be a powerful tool for pooling results from different studies to overcome the issue of small sample size (Barber, Bennett et al. 2007). Based on the results of the present meta-analysis, an association between rs7903146 and PCOS seems unlikely. Even so, the present population of 1892 women with PCOS and 2695 controls is smaller than that of many genetic studies comparing effect sizes, which is a limitation. However, all the patients genotyped for *TCF7L2* SNP and reported in the literature were included in this meta-analysis.

Another potential association that remains to be confirmed is that of SNPs rs11196236 and rs11196229 on *TCF7L2* gene with PCOS (Biyasheva, Legro et al. 2009). One previous study failed to confirm this association (Ramos, Wiltgen et al. 2013), while another found an association only with peripheral insulin resistance in PCOS (Yalamanchi, Sam et al. 2012). However, not enough studies are available in the literature regarding PCOS patients to allow a meta-analysis to be conducted.

Indeed, the decision to study only the rs7903146 polymorphism was based on the fact that only a few studies with the rs12255372 polymorphisms are available in literature, limiting the possibility to perform a meta-analysis. At the time the present data extraction was completed (August 2014), only three studies reporting the rs12255372 in Asian (Kim, Choi et al. 2012), European (Vcelak, Vejrazkova et al. 2012) and African PCOS (Ben-Salem, Ajina et al. 2013) patients had been published.

The studies included in this meta-analysis were highly consistent, with no evidence of heterogeneity between populations and no publication bias. Another important point is that all studies included in the meta-analysis used the Rotterdam criteria to diagnose PCOS, ensuring a homogeneous PCOS population.

In conclusion, the present results suggest that the rs7903146 T allele is not associated with risk for PCOS in non-Asian or Asian women.

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Table 1. Characteristics of studies in the meta-analysis

Name Year	Ethnicity (country)	PCOS criteria	Cases[a]/Controls[b]	Age (cases[a]/controls[b])	BMI (cases[a]/controls[b])	Genotypes (cases[a]/controls[b])		
						CC	CT	TT
Barber 2007	European (Uk British/Irish)	Rotterdam	358	32 (7.2)	26.6 (NR)	177	151	30
			1243	NR	NR	600	539	104
Christopoulos 2008	European (Greece)	Rotterdam	183	23.9 (5.17)	27.3 (6.77)	43	108	32
			148	26.2 (7.08)	25.8 (7.27)	52	76	20
Kim 2012	Asian (Korea)	Rotterdam	377	28.5 (4.9)	22.2 (4.0)	350	27	0
			386	28.9 (4.5)	20.9 (3.1)	357	29	0
Xu 2010	Asian (China)	Rotterdam	326	26.1 (4.0)	22.5 (4.0)	261	61	4
			290	29.1 (5.1)	21.4 (2.2)	232	56	2
Vcelák 2012	European (Czech Republic)	Rotterdam	329	27.5 (6.3)	27.0 (6.6)	178	127	24
			376	29.9 (10.8)	23.3 (4.4)	204	147	24
Ramos 2013	Caucasian (Brazil)	Rotterdam	200	22.8 (6.6)	30.2 (7.1)	104	55	33
			102	25.2 (7.6)	27.0 (6.0)	51	36	10
A. Ben-Salem 2014	Tunisian (Tunisia)	Rotterdam	119	29.8 (4.7)	28.4 (7.1)	37	51	30
			150	30.6 (5.9)	24.5 (3.8)	46	69	32

Mean age and BMI are shown with SD in parentheses

NR: Not reported.

[a] top row; [b] bottom row

Table 2. Newcastle-Ottawa quality (NOS) assessment scale for the studies included in the meta-analysis

Author	Year	Selection	Comparability	Exposure
Barber	2007	***	*	**
Christopoulos	2008	***	*	**
Kim	2012	***	*	**
Xu	2010	***	*	*
Vcelák	2012	***	*	*
Ramos	2013	****	*	**
Ben-Salen	2013	****	*	**

Quality of selection (minimum 1- maximum 4 stars)
Comparability (minimum 0- maximum 1star)
Exposure (minimum 1- maximum 3 stars)

Figure Legends

Figure 1. PRISMA flow diagram of the study selection process

Figure 2. Subgroup analysis: pooled odds ratios of PCOS and rs7903146 (“A” allele contrast model, “B” additive model, “C” dominant model, “D” recessive model)

Figure 3. Funnel plot with 95% confidence intervals (“A” allele contrast model, “B” additive model, “C” dominant model, “D” recessive model)

Figures

Figure I. PRISMA flow diagram of the study selection process

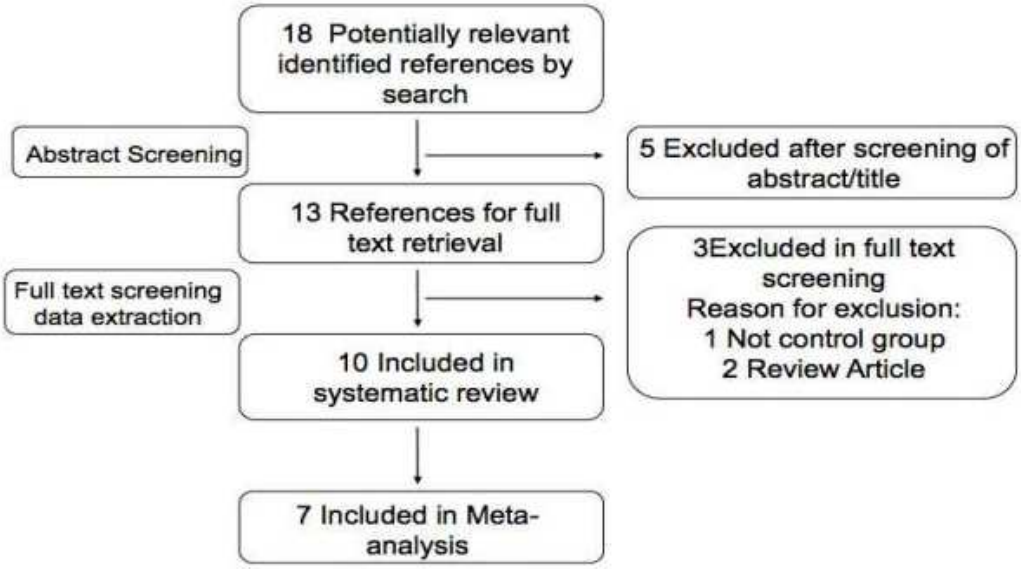


Figure 2

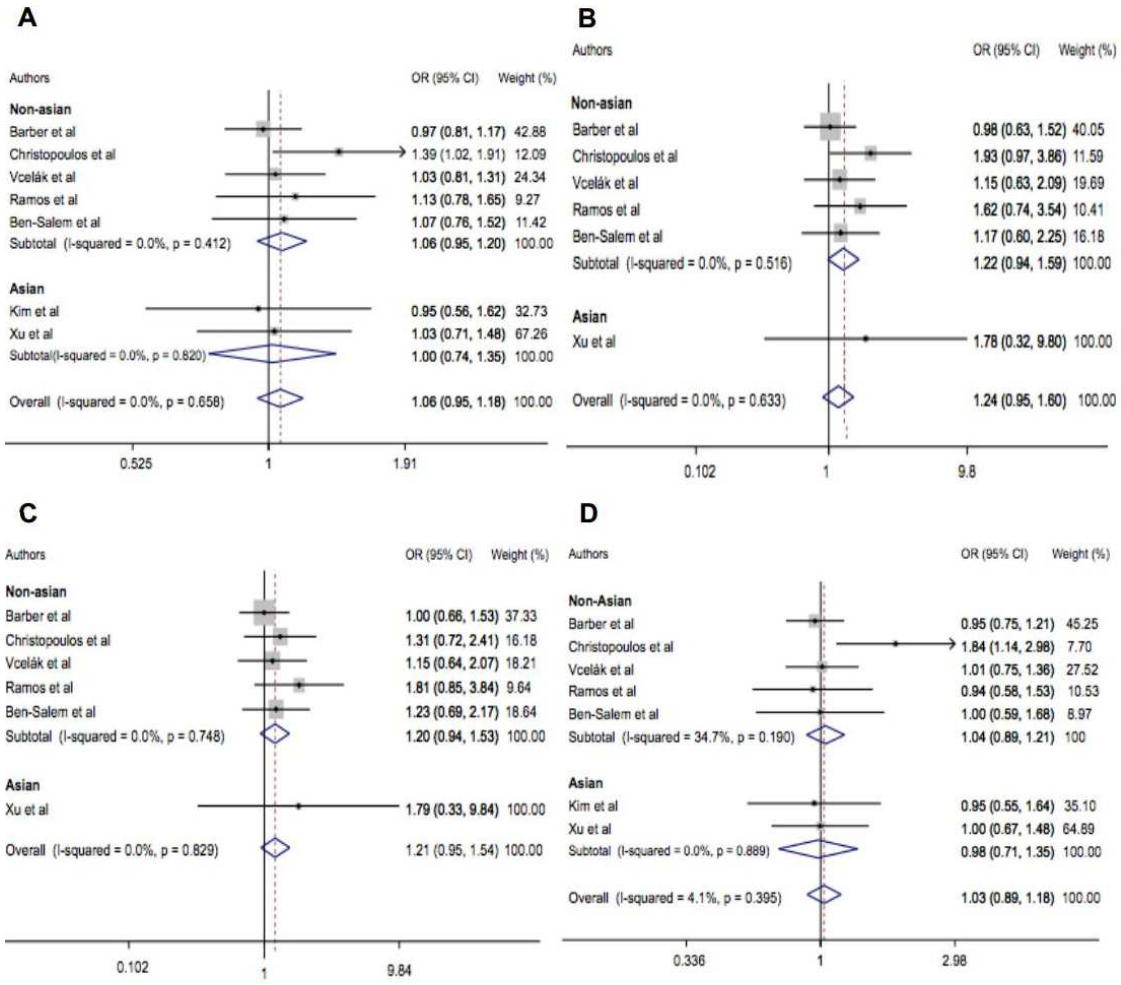
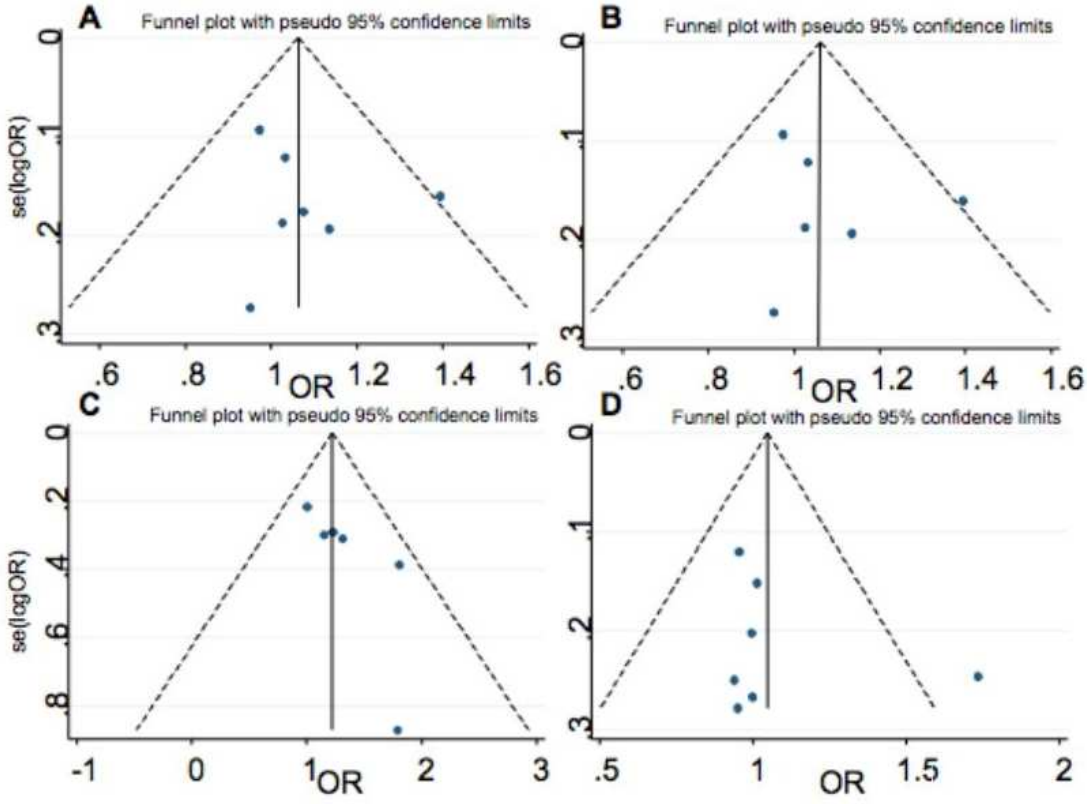

A
Authors
OR (95% CI)
Weight (%)
Non-asian
Barber et al
0.97 (0.81, 1.17) 42.88
Christopoulos et al
1.39 (1.02, 1.91) 12.09
Vcelák et al
1.03 (0.81, 1.31) 24.34
Ramos et al
1.13 (0.78, 1.65) 9.27
Ben-Salem et al
1.07 (0.76, 1.52) 11.42
Subtotal (I-squared = 0.0%, p = 0.412)
1.06 (0.95, 1.20) 100.00
Asian
Kim et al
0.95 (0.56, 1.62) 32.73
Xu et al
1.03 (0.71, 1.48) 67.26
Subtotal(I-squared = 0.0%, p = 0.820)
1.00 (0.74, 1.35) 100.00
Overall (I-squared = 0.0%, p = 0.658)
1.06 (0.95, 1.18) 100.00
0.525
1
1.91
B
Authors
OR (95% CI)
Weight (%)
Non-asian
Barber et al
0.98 (0.63, 1.52) 40.05
Christopoulos et al
1.93 (0.97, 3.86) 11.59
Vcelák et al
1.15 (0.63, 2.09) 19.69
Ramos et al
1.62 (0.74, 3.54) 10.41
Ben-Salem et al
1.17 (0.60, 2.25) 16.18
Subtotal (I-squared = 0.0%, p = 0.516)
1.22 (0.94, 1.59) 100.00
Asian
Xu et al
1.78 (0.32, 9.80) 100.00
Overall (I-squared = 0.0%, p = 0.633)
1.24 (0.95, 1.60) 100.00
0.102
1
9.8
C
Authors
OR (95% CI)
Weight (%)
Non-asian
Barber et al
1.00 (0.66, 1.53) 37.33
Christopoulos et al
1.31 (0.72, 2.41) 16.18
Vcelák et al
1.15 (0.64, 2.07) 18.21
Ramos et al
1.81 (0.85, 3.84) 9.64
Ben-Salem et al
1.23 (0.69, 2.17) 18.64
Subtotal (I-squared = 0.0%, p = 0.748)
1.20 (0.94, 1.53) 100.00
Asian
Xu et al
1.79 (0.33, 9.84) 100.00
Overall (I-squared = 0.0%, p = 0.829)
1.21 (0.95, 1.54) 100.00
0.102
1
9.84
D
Authors
OR (95% CI)
Weight (%)
Non-Asian
Barber et al
0.95 (0.75, 1.21) 45.25
Christopoulos et al
1.84 (1.14, 2.98) 7.70
Vcelák et al
1.01 (0.75, 1.36) 27.52
Ramos et al
0.94 (0.58, 1.53) 10.53
Ben-Salem et al
1.00 (0.59, 1.68) 8.97
Subtotal (I-squared = 34.7%, p = 0.190)
1.04 (0.89, 1.21) 100
Asian
Kim et al
0.95 (0.55, 1.64) 35.10
Xu et al
1.00 (0.67, 1.48) 64.89
Subtotal (I-squared = 0.0%, p = 0.889)
0.98 (0.71, 1.35) 100.00
Overall (I-squared = 4.1%, p = 0.395)
1.03 (0.89, 1.18) 100.00
0.336
1
2.98

Figure 3


A
Funnel plot with pseudo 95% confidence limits
se(logOR)
0
.1
.2
.3
.6
.8
1
OR
1.2
1.4
1.6
B
Funnel plot with pseudo 95% confidence limits
se(logOR)
0
.1
.2
.3
.6
.8
1
OR
1.2
1.4
1.6
C
Funnel plot with pseudo 95% confidence limits
se(logOR)
0
.2
.4
.6
.8
-1
0
1
OR
2
3
D
Funnel plot with pseudo 95% confidence limits
0
.1
.2
.3
.5
1
OR
1.5
2

Parte 3

FTO gene Variants are not Associated with Women with Polycystic Ovary Syndrome from Southern Brazil

FTO gene variants are not associated with women with Polycystic ovary syndrome from Southern Brazil

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Abstract

Background and Aims: Polycystic ovary syndrome (PCOS) is a common endocrine disorder, presenting polygenic traits as well as determined by environmental factors. Given the overlap between PCOS and obesity, we assessed the frequencies of SNPs rs9939609 and rs8050136 in intron 1 of the *FTO* gene and their haplotypes in women with PCOS and healthy controls with regular cycles from Southern Brazil and investigated their relationship with metabolic traits and endocrine parameters.

Subjects and Methods: The sample comprised 298 women (199 with PCOS and 99 non-hirsute women with regular ovulatory cycles). *FTO* genotyping was done by real-time PCR. Haplotypes were constructed from the combination of both polymorphisms. Frequencies were inferred using PHASE 2.1.1 software.

Results: The distribution of rs9939609 (PCOS: 32.6% TT, 45.9% TA, 21.5% AA; controls: 33.3% TT, 49.0% TA, 17.7% AA) and rs8050136 (PCOS: 21.7% AA, 43.3% AC, 35.0% CC; controls: 14.9% AA, 48.9% AC, 36.2% CC) was similar between groups. The mean age of participants was 22.7 ± 7.1 years. Women with PCOS had significantly higher BMI, waist circumference, total testosterone, and FAI vs. controls. In the PCOS group, no differences between genotypes and haplotypes were found for clinical variables. Presence of at least one risk allele for polymorphisms rs9939609 and rs8050136 was associated with higher fasting glucose levels.

Conclusion: Our findings indicate that neither the *FTO* rs9939609 and rs8050136 polymorphisms nor its haplotypes are related to PCOS, but suggest an association between the presence of risk alleles of SNPs rs9939609 and rs8050136 in *FTO* and glucose levels in women from Southern Brazil.

Keywords: *FTO* gene, single nucleotide polymorphism, polycystic ovary syndrome, glucose

1. Introduction

Polycystic ovary syndrome (PCOS) is a heterogeneous disease characterized mainly by chronic anovulation and manifestations of hyperandrogenism. Using the Rotterdam consensus diagnostic criteria, PCOS affects up to 18% (March et al. 2009). Obesity is one of the most prevalent features of PCOS (Graff et al. 2013; Peppard et al., 2001; ESHRE/ASRM 2004; Apridonidze et al., 2005). A recent meta-analysis showed that women with PCOS had increased prevalence of overweight as well as obesity (Lim et al. 2012), and abdominal obesity is found in most women with the condition, in association with metabolic disturbances such as insulin resistance (IR) (Apridonidze et al., 2005). Indeed, the metabolic syndrome (MetS), defined as a cluster of central obesity, dyslipidemia, hypertension, and glucose intolerance, is found in 30-40% of women with PCOS, and 25-35% of obese women with PCOS are diagnosed with impaired glucose tolerance or diabetes around the age of 30. This prevalence is nearly twofold higher than that for age-adjusted women in the general population (Gambineri et al., 2002; Apridonidze et al., 2005; Carmina et al., 2006; Wild et al. 2010). In general, women with PCOS have 3-7 times more risk of developing diabetes mellitus type 2 (DM2) compared with the general population (Ehrmann et al., 1999; Legro et al., 1999).

Individual susceptibility to PCOS is determined by genetic and environmental factors. Among several candidate genes for obesity, the fat mass and obesity associated gene (*FTO*) has been recently identified. *FTO* is located on chromosome 16q12.2 (Frayling et al., 2007) and, statistically, remains the candidate gene with the largest effect size on obesity. The protein it encodes is a member of the nonheme dioxygenase (Fe(II)- and 2-oxoglutarate-dependent dioxygenases) superfamily (Gerken et al., 2007; Sanchez-Pulido and Andrade-Navarro, 2007), and is involved in various cellular processes, including DNA repair, fatty acid metabolism, and posttranslational modifications. However, to date, its link to body weight regulation remains unclear.

Thus far, studies have shown controversial results. Some investigations demonstrated an association with PCOS (Attaoua et al., 2008; Barber et al.,

2008; Yan et al., 2009), whereas others found no such relation (Ewens et al. 2011; Kim et al. 2014). This difference may be due to factors such as ethnicity or whether there was adjustment for body mass index (BMI) in each individual study.

A recent meta-analysis showed no association between *FTO* gene polymorphisms and PCOS susceptibility. On subgroup analysis, the association was significant in East Asians but not in Caucasians, suggesting an effect of ethnicity on the association. Further studies are required to assess the real impact of polymorphisms in the *FTO* gene on PCOS in other populations (Cai et al., 2014).

Within this context, the aim of the present study was to assess whether the prevalence of SNPs rs9939609 and rs8050136 in intron 1 of the *FTO* gene and their haplotypes differs between women with PCOS and healthy controls with regular cycles from Southern Brazil, and to investigate the relationship of these genetic factors with metabolic traits and endocrine parameters.

2. Material and methods

2.1 Patients

The study population comprised 298 women of reproductive age: 199 patients with PCOS and 99 non-hirsute women with regular, ovulatory cycles (luteal phase progesterone >3.8ng/ml). All participants had attended a university hospital in Southern Brazil (Hospital de Clínicas de Porto Alegre, state of Rio Grande do Sul) or were recruited by an advertisement placed in the local media between 2009 and 2013. PCOS was diagnosed according to the Rotterdam criteria as the presence of two out of three of the following signs: 1) Oligoamenorrhea and/or chronic anovulation (≤ 9 cycles/year and/or luteal phase progesterone < 3.8 ng/mL), 2) clinical and/or biochemical hyperandrogenism, and 3) polycystic ovaries (PCO) on ultrasound examination. Diagnosis of PCOS also relied on exclusion of other hyperandrogenic disorders (18). None of the PCOS or control participants had received any drugs known to interfere with hormone levels (such as oral contraceptive pills, antiandrogens, metformin, fibrates, or statins) for at least 3 months before the study. The exclusion criteria were pregnancy, liver disease, or kidney disease.

2.2 Study protocol

Anthropometric measurements included body mass index (BMI) (current weight in kg divided by the height in m squared) and waist circumference (measured at the midpoint between the lower rib margin and the iliac crest) (Toscani et al., 2007). Blood pressure was measured after a 10-minute rest, with the patient seated, with both feet on the floor, and the arm supported at heart level. Two measurements were obtained 10 minutes apart using an Omron HEM-742INT automatic blood pressure monitor (Rio de Janeiro, Brazil) with the correct cuff size for the arm diameter. MetS and the cutoffs for its isolated components were defined in accordance with the Joint Scientific Statement (Alberti et al., 2009).

Approval for this study was obtained from the Institutional Review Board and the local Research Ethics Committee, functioning according to the 3rd edition of the Guidelines on the Practice of Ethical Committees in Medical Research issued by the Royal College of Physicians of London, and written informed consent was obtained from every subject.

2.3 Laboratory measurements

All samples were obtained between 8:00 and 10:00 am, after a 12-h overnight fast. Blood samples were drawn from an antecubital vein for determination of hormonal levels. Glucose levels were determined by enzymatic colorimetric methods (Bayer 1650 Advia System). Hormonal measurements were performed using commercial kits, as previously described (Nacul et al., 2007; Toscani et al., 2007; Wiltgen et al., 2009). The free androgen index was calculated as *testosterone (nmol/L) / SHBG (nmol/L) x 100*.

2.4 Genotyping

In addition to serum samples, whole blood samples were collected from all participants. Genomic DNA was extracted from peripheral leukocytes as reported elsewhere (Ramos et al., 2013). The DNA samples were diluted to 2

ng/mL and genotyped for SNP rs9939609 T>A and rs8050136 A>C of the *FTO* gene by real-time polymerase chain reaction (7500 Fast Real-Time Polymerase Chain Reaction System, Applied Biosystems, CA, USA), using the allelic discrimination assay with TaqMan MGB primers and probes (Applied Biosystems, CA, USA). TaqMan Master mix (2.5 μL), TaqMAn assay (0.25 μL), and H_2O (1.25 μL) were added for a final volume of 4 μL per sample, and 1 μL of DNA was added for a total reaction volume of 5 μL. Reaction conditions for the SNP rs9939609 were: 10 min at 95°C after 50 cycles of denaturation at 95°C (15 s) and annealing at 61°C (1 min). Endpoint fluorescent readings were performed in the 7500 Fast System Sequence Detection Software version 1.4 environment. The internal quality of genotype data was assessed by typing 20% of blinded samples in duplicate.

Haplotypes were constructed from the combination of the two *FTO* polymorphisms (rs9939609 and rs8050136), and their frequencies were inferred using the PHASE 2.1.1 program (Stephens et al., 2001). The first letter of each haplotype refers to the rs8050136 polymorphism, and the second to the rs9939609 polymorphism.

2.5 Statistical analysis

Results are presented as mean ± standard deviation for normally distributed variables or as median (interquartile range) for variables with a non-Gaussian distribution. The Kolmogorov–Smirnov test and descriptive statistics were used to evaluate the distribution of data. Non-Gaussian variables were log-transformed for statistical analysis with an independent Student *t* test and one-way ANOVA and reported as back-transformed into their original units. Binary logistic regression analyses were performed to examine the ORs of PCOS (dependent variable) with genotype. The chi-square test was used to compare categorical variables, and to assess deviation of the genotype frequencies from Hardy–Weinberg equilibrium. Previous studies have shown that the rs8050136 “A” allele is in strong linkage disequilibrium with the rs9939609 “A” allele (Frayling et al., 2007; Hotta et al., 2008; Ramos et al., 2011). For each studied polymorphism, patients were divided into two groups:

one combined heterozygous and risk homozygous subjects into a single group and the second comprised non-risk homozygous subjects. For rs9939609, we combined TA (heterozygous) and AA (polymorphic homozygous) as one group and wild type TT (wild homozygous) as the second group, with a dominant model; for rs8050136, we combined AC (heterozygous) and AA (wild homozygous) as one group and CC (polymorphic homozygous) as the second group, with a recessive model. All analyses were carried out in SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA). Findings were considered significant at $p<0.05$.

3. Results

Participants were mostly Caucasian (93.9%), with the remaining participants having mixed African and European ancestry. The mean age of participants was 22.7 ± 7.1 years. The clinical characteristics of the sample are shown in Table 1. As expected, women with PCOS had significantly higher BMI, waist circumference, total testosterone and FAI ($p= 0.001$), as well as a higher frequency of blood pressure (BP) ≥ 130/85 mmHg, HDL-C < 50 mg/dL, triglycerides ≥ 150mg/dL, waist circumference > 88 cm, and MetS when compared with the control group ($p\leq0.05$; Table 1).

The genotypes of the entire sample and its subgroups were in Hardy-Weinberg equilibrium. Most of the samples (96.9% in the control group and 91% in the PCOS group) were successfully genotyped. Genotype frequencies for rs9939609 in the *FTO* gene were as follows: 33.3% TT, 49% TA, and 17.7% AA for controls and 32.6% TT, 45.9% TA, and 21.5% AA for the PCOS group. Genotype frequencies for rs8050136 were as follows: 14.9% AA, 48.9% AC, and 36.2% CC for controls and 21.7% AA, 43.3% AC, and 35% CC for PCOS subjects. Six haplotypes were inferred in this sample (Ht1: CTCT, Ht2: CTCA, Ht3: CTAA, Ht4: CAAA, Ht5: ATAA, Ht6: AAAA). Haplotype frequencies were 33.7% for Ht1, 50.0% for Ht2/Ht3/Ht4/Ht5, and 16.3% for Ht6 in the control group, versus 32.1% for Ht1, 48.4% for Ht2/Ht3/Ht4/Ht5, and 19.6% for Ht6 in the PCOS group.

Logistic regression analysis demonstrated that SNPs rs9939609 and rs8050136 and their haplotypes were not associated with an increased risk of

PCOS (ORs 1.03 (0.61 – 1.74), 1.05 (0.62 – 1.77) and 1.07 (0.63 – 1.81), respectively; all p>0.05).

No interaction between cases and controls in the studied variables was observed among genotypes. Therefore, subsequent analyses were conducted specifically on the PCOS group. Table 3 shows BMI and biochemical characteristics according to the different SNPs and haplotypes in PCOS subjects. BMI and hormone levels (total testosterone and FAI) were similar between genotypes, as were the frequencies of MetS. In turn, individuals with at least one risk allele, TA (heterozygous) and AA (polymorphic homozygous) genotypes, had significantly higher glucose levels compared with individuals presenting the wild TT genotype for SNP rs9939609 (TA/AA: 91.5 ± 18.4 vs. TT: 85.8 ± 11.5, p=0.03). For SNP rs8050136, women with the wild AA genotype and heterozygous AC also exhibited higher fasting glucose levels (AA/AC: 91.7 ± 18.7 vs. CC: 86.5 ± 10.9, p=0.04), while haplotypes presented a trend toward higher fasting glucose (Ht2/Ht3/Ht4/Ht5/Ht6: 91.0 ± 18.5 vs. 86.4 ± 11.0 Ht1, p=0.08) (Figure 1).

4. Discussion

The present study suggests that the *FTO* gene polymorphisms rs9939609 and rs8050136, as well as haplotypes, are not associated with risk of PCOS. This is the first study to evaluate such an association in a Southern Brazilian population. Previous studies with PCOS populations did not show conclusive results; three demonstrated that variation within the *FTO* gene is significantly associated with PCOS in UK, Chinese, and Korean populations, but these associations were attenuated and no longer significant after adjusting for age and BMI (Barber et al., 2008; Yan et al., 2009; Song do et al. 2008). Other studies found no association with PCOS and the *FTO* gene, but have found an association between PCOS and metabolic traits such as BMI, body weight, and fat mass (Attaoua et al., 2008; Tan et al. 2010; Wehr et al. 2010; Kim et al. 2014). These results are corroborated by a recent meta-analysis, which demonstrated that the rs9939609 polymorphism (or its proxy) was not related to PCOS susceptibility after adjustment for BMI (Cai et al., 2014). This meta-analysis suggested there might be a direct association between *FTO* variants

and PCOS risk specifically in an East Asian population, since the association was significant after adjusting for BMI (Cai et al. 2014). No studies are available concerning polymorphisms in the *FTO* gene and PCOS in Latin American populations.

Furthermore, in this study we observed no differences in total testosterone levels nor in FAI value across carriers of the various genotypes. These results are consistent with previous studies. One investigation conducted in women from the UK showed no relationship between *FTO* and androgen levels (Barber et al., 2008). A second study conducted in a Polish population did not find differences in testosterone, SHBG, and FAI between different genotypes (Kowalska et al., 2009). Finally, a recent study on rs8050136 and two other polymorphisms showed no association with hyperandrogenemia after adjusting for BMI (Song do et al. 2008). Only one study has reported higher free testosterone in the presence of allele A of polymorphism rs9939609 (Wehr et al. 2010).

In the present study, both risk alleles for polymorphisms rs9939609 and rs8050136 were found to be associated with higher glucose levels. A previous study in patients with PCOS also found that impaired fasting glucose was associated with the rs1421085 (C/T) polymorphism in a Central European population, independently of BMI or age (Attaoua et al., 2008). In addition, while few studies in patients with PCOS failed to find an association with glucose levels (Kowalska et al.; Kim et al., 2014; Song do et al., 2014), one study assessing the rs9939609 *FTO* variant and 10 metabolic traits using data from 17,037 white European individuals revealed evidence for an association between *FTO* genotype and fasting insulin, glucose, triglycerides, and HDL cholesterol (Freathy et al., 2008). Recently, our group also confirmed the association between *FTO* genotypes and higher glucose levels in menopausal women (Ramos et al., 2011).

Regarding MetS, our study suggests that neither of the tested SNPs nor any of the haplotypes of *FTO* are major risk factors for the syndrome, confirming a previous study in a population of 457 obese Caucasian females (de Luis et al., 2013).

To date, there is no known mechanism linking the *FTO* gene directly to PCOS. In fact, studies in which an association was found between *FTO* and

PCOS susceptibility also observed an increase in adiposity, which could be linked to a genetic interaction of the *FTO* gene with other susceptibility genes, combined to create the polygenic background of PCOS (Kowalska et al., 2009). It is also important to point out that the studied polymorphisms, which are located in intron 1, are in linkage disequilibrium, making it difficult to discern the best single SNP surrogate to fully capture genetic variability for this region.

5. Conclusion

In conclusion, the present study was the first to assess *FTO* gene variants and PCOS susceptibility in Brazilian women. Our results indicated that neither the *FTO* polymorphisms rs9939609 and rs8050136 nor its haplotypes were related to PCOS, but suggest an association between the presence of risk alleles of SNPs rs9939609 and rs8050136 and glucose levels in women from southern Brazil.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Tables

Table 1. Clinical and biochemical profile of the sample

Variable	PCOS (n=199)	Controls (n=99)	P-value
BMI (kg/m²)	29.6 ± 6.4	27.0 ± 6.0	0.001
Waist circumference (cm)	89.2 ± 15.0	78.1 ± 11.5	0.001
Fasting glucose (mg/dL)	88.8 ± 12.2	88.4 ± 7.5	0.78
Total testosterone (nmol/L)	0.9 ± 0.4	0.5 ± 0.1	0.001
FAI	3.40 (1.95 – 5.95)	1.25 (0.93 – 2.02)	0.001
Glucose ≥100 mg/dL (%)	19 (9.7%)	6 (7.7%)	0.60
BP ≥130/85 mmHg (%)	68 (35.1%)	6 (7.1%)	0.005
HDL-C <50 mg/dL (%)	112 (56.6%)	35 (40.7%)	0.014
Triglycerides ≥150mg/dL (%)	35 (17.8%)	4 (4.7%)	0.003
Waist >88 cm (%)	91 (49.2%)	12 (16.2%)	0.001
Metabolic syndrome	49 (24.6%)	2 (2.4%)	0.001

Data are expressed as mean ± SD or median (interquartile range) (Student *t* test). Total testosterone reference range (female): 0.2-0.8 ng/mL; metabolic syndrome and its isolated components are presented as number of positive cases and percentage. BMI, body mass index; FAI, free androgen index; BP, blood pressure.

Table 2. Association between *FTO* gene variants and PCOS susceptibility

	Risk/non-risk allele	Controls	PCOS	p
rs9939609	A/T			
TT		33.3%	32.6%	
TA/AA		66.7%	67.4%	
		OR 1.03 (0.61 – 1.74)		0.90
rs8050136	A/C			
AA/AC		63.8%	65.0%	
CC		36.2%	35.0%	
		OR 1.05 (0.62 – 1.77)		0.84
Haplotypes	AA/CT			
Ht1		33.7%	32.1%	
Ht2/Ht3/Ht4/Ht5/Ht6		66.3%	67.9%	
		OR 1.07 (0.63 – 1.81)		0.78

Table 3. Clinical characteristics of the PCOS group according to different genotypes for SNP rs9939609, rs8050136 and haplotypes.

rs9939609		**PCOS**		**p[a]**	**p[b]**
	TT	TA	AA		
BMI	29.2 ± 6.7	30.52 ± 7.3	30.6 ± 7.1	0.51	0.25
TT (nmol/L)	0.86 ± 0.36	0.90 ± 0.42	0.99 ± 0.50	0.34	0.42
FAI	3.48 (1.86 – 5.33)	2.81 (1.94 – 5.97)	4.68 (1.79 – 6.93)	0.28	0.85
MetS (%)	11 (24.4%)	25 (55.6%)	9 (20.0%)	0.29	0.17
rs8050136					
	AA	AC	CC		
BMI	30.9 ± 7.2	30.6 ± 7.4	29.1 ± 6.7	0.34	0.15
TT (nmol/L)	0.99 ± 0.50	0.90 ± 0.41	0.86 ± 0.36	0.30	0.41
FAI	4.62 (1.84 – 6.82)	2.83 (2.04 – 6.00)	3.28 (1.83 – 5.30)	0.40	0.91
MetS (%)	8 (17.8%)	25 (55.6%)	12 (26.7%)	0.15	0.17
Haplotypes					
	Ht1	Ht2/Ht3/Ht3/Ht5	Ht6		
BMI	29.5 ± 6.8	30.3 ± 7.2	30.6 ± 7.4	0.72	0.43
TT (nmol/L)	0.86 ± 0.36	0.92 ± 0.42	0.98 ± 0.49	0.46	0.42
FAI	3.63 (1.88 – 5.32)	2.81 (1.94 – 5.97)	4.68 (1.90 – 6.71)	0.29	0.80
MetS (%)	12 (26.1%)	26 (56.5%)	8 (17.4%)	0.43	0.66

Date are expressed as mean ± SD values or median (interquartile range) (one-way ANOVA or Student *t* test); BMI, body mass index; FAI, free androgen index; TT, total testosterone; MetS, metabolic syndrome. [a] Between carriers of TT vs TA vs AA genotype for rs9939609; AA vs AC vs CC for rs8050136; and Ht1 vs Ht2/Ht3/Ht3/Ht5 vs Ht6 for haplotypes . [b] Between carriers of TT vs TA/AA genotype for rs9939609; AA/AC vs CC for rs8050136; and Ht1 vs Ht2/Ht3/Ht3/Ht5/Ht6 for haplotypes.

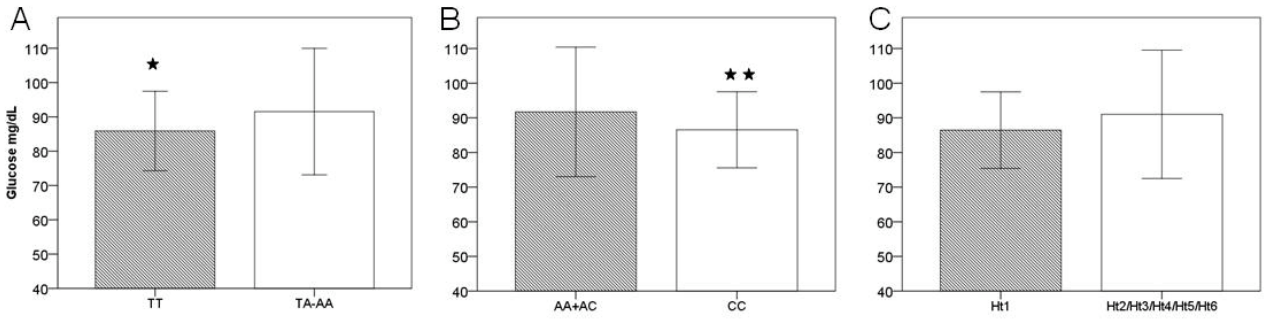

Figure Legend

Figure 1. Associations of SNP rs9939609 (**A**), SNP rs8050136 (**B**) and haplotypes (**C**) with glucose. (Student's t-test). *p=0.03; **p=0.04

Considerações Finais

No presente estudo demonstramos que 4 alterações genéticas específicas nos genes *TCF7L2* e *FTO* não estão associados com o fenótipo de PCOS. Além de disso, realizamos uma meta análise para o polimorfismo rs7903146 do gene do *TCF7L2* que corroborou com nosso estudo caso controle. Adicionalmente, observamos que pacientes portadoras dos alelos polimórficos rs7903146 e rs11196236 apresentam uma tendência de maior circunferência da cintura, LDL-c e colesterol total. De forma interessante, no gene do *FTO* nossos resultados sugerem que pacientes portadoras do genótipo de risco dos polimorfismos rs9939609 e rs8050136 apresentam maiores níveis de glicose sérica.

Estes dados em conjunto sugerem que a presença dos polimorfismos em genes ligados a obesidade e DM2 não estão associados com uma maior prevalência de PCOS, mas estes SNPs podem contribuir com as diferenças metabólicas das pacientes. Além disso estes resultados ampliam os conhecimentos sobre as bases moleculares da PCOS.

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