UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

CURSO DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS:

ENDOCRINOLOGIA

O Papel da Desiodase Tipo 2 na Resistência à Insulina

Jose Miguel Dora

Tese de Doutorado

Porto Alegre, outubro de 2011.

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Tese apresentada como requisito parcial para obtenção do título de Doutor em Endocrinologia, à Universidade Federal do Rio Grande do Sul, Programa de Pós-Graduação em Ciências Médicas: Endocrinologia.

Orientadora: Profa. Dra. Ana Luiza Maia

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Este trabalho é dedicado

Ao Gabriel e à Betânia, minhas fontes de energia renovável.

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Esta Tese de Doutorado segue o formato proposto pelo Programa de Pós-Graduação em Ciências Médicas: Endocrinologia, Faculdade de Medicina, UFRGS, sendo apresentada na forma de 01 Resumo da Tese, 01 artigo de revisão e 02 trabalhos originais publicados e/ou submetidos à publicação:

1. Resumo da Tese

2. Artigo de revisão geral do tema: “Breve revisão sobre o papel do metabolismo dos hormônios da tireóide na resistência à insulina.” (a ser enviado para publicação).

3. Artigo Original: “Association of the type 2 deiodinase Thr92Ala polymorphism with type 2 diabetes: case-control study and meta-analysis” (*Eur J Endocrinol.* 2010; 163(3): 427-34).

4. Artigo Original: “The type 2 deiodinase Thr92Ala polymorphism is associated with disrupted placental activity but not with dysglycemia or adverse gestational outcomes” (em processo de resposta aos revisores no periódico *Fertility and Sterility*).

Resumo da Tese

Os hormônios tireoideanos têm importante papel na manutenção da homeostase metabólica, exercendo efeitos sobre o metabolismo glicêmico em diferentes níveis. Alterações nos níveis de hormônios tireoideanos, como ocorrem no hipertireoidismo e no hipotireoidismo, estão associados a aumento da resistência à insulina. A captação de glicose mediada pela insulina no músculo esquelético e no tecido adiposo, um dos fatores determinantes da homeostase glicêmica, é dependente da expressão do transportador de glicose tipo 4 (GLUT-4) na membrana celular destes tecidos. Visto que o gene do GLUT-4 é induzido pelos hormônios tireoideanos, alterações na concentração de triiodotironina (T3) tecidual podem explicar a associação entre alterações nos níveis de hormônios tireoideanos e resistência à insulina. No músculo esquelético a ativação do pro-hormônio tiroxina (T4) ao hormônio ativo T3 é regulada através da atividade da enzima desiodase tipo 2 (D2). Estudos em modelos animais nocaute para o gene da enzima D2 (D2K0) demonstraram resistência à insulina aumentada nestes animais. Em humanos, um polimorfismo da D2, no qual uma treonina (Thr) é trocada por uma alanina no codon 92 (D2 Thr92Ala), foi associado a redução da atividade da D2 em tecidos periféricos e maior resistência à insulina em pacientes com e sem diabetes mellitus tipo 2 (DM2).

A associação entre o polimorfismo Thr92Ala da D2 e aumento de risco para DM2 não foi demonstrada nos estudos que avaliaram esta questão. Entretanto, visto que DM2 é uma doença poligênica e que os marcadores genéticos para esta doença tem tamanho de efeito pequeno, deve-se

considerar falta de poder estatístico como uma possibilidade para explicar os resultados negativos.

No primeiro artigo original que compõe esta Tese o objetivo foi avaliar se o polimorfismo Thr92Ala da D2 está associado com aumento de risco para DM2. Para tanto, planejou-se um estudo de caso-controle seguido de uma revisão sistemática e meta-análise da literatura. No estudo de caso-controle, foram incluídos 1057 pacientes com DM2 e 516 indivíduos controles saudáveis. A prevalência do genótipo Ala92Ala da D2 foi de 16,4% no grupo DM2 e 12,0% no grupo controle (p=0,03), resultando em uma razão de chances (RC) de 1,41 (IC95% 1,03-1,94, p=0,03). No mesmo trabalho realizou-se uma revisão sistemática com meta-análise de estudos observacionais que avaliaram a associação entre o genótipo Ala92Ala da D2 e DM2, sendo incluídos 4 estudos. A meta-análise dos 4 estudos, incluindo dados de um total de 11.033 pacientes, identificou uma RC 1,18 (IC95% 1,03-1,35, p=0,02) para a associação do genótipo Ala92Ala da D2 e DM2. Portanto, os resultados do estudo de caso-controle e da meta-análise demonstraram que o genótipo Ala92Ala da D2 está associado a aumento de risco para DM2 na população geral.

A gestação caracteriza-se por uma série de alterações hormonais. Com respeito aos hormônios tireoideanos, há um aumento na produção e metabolização do T4. No metabolismo glicêmico, especialmente no terceiro trimestre, há um estado de resistência à insulina, induzido pela secreção de uma série de hormônios contra-reguladores da insulina pela placenta. A placenta também regula a transferência dos hormônios tireoideanos da mãe para o feto através da expressão das enzimas D2 e desiodase tipo 3 (D3).

Portanto, em um contexto de resistência à insulina e de aumento de demanda pela produção de T4, um polimorfismo que está associado a redução da atividade da D2 em tecidos periféricos e maior resistência à insulina, pode associar-se a pior controle glicêmico durante a gestação. Portanto, o objetivo do segundo artigo original que compõe esta Tese foi de avaliar a associação entre o genótipo Ala92Ala da D2 e o controle glicêmico em gestantes. Neste estudo foram incluídas 110 gestantes (19 Ala92Ala e 91 Thr92Ala-Thr92Thr), que foram acompanhadas até o parto. Não foram identificadas diferenças no controle glicêmico ao longo da gestação, no peso neonatal ou em desfechos obstétricos entre os dois grupos. Em 30 amostras de placentas obtidas no momento do parto, realizou-se ensaios para avaliar a expressão da enzima D2. A atividade placentária da D2 das pacientes Ala92Ala foi 82% menor que nos demais genótipos ($p<0,001$).

Pelas potenciais implicações terapêuticas no futuro, o entendimento dos mecanismos que explicam a associação entre redução na atividade da D2 e resistência à insulina e aumento do risco de DM2 são de especial interesse.

Breve revisão sobre o papel do metabolismo dos hormônios da tireóide na resistência à insulina.

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Resumo

Os hormônios tireoideanos têm importante papel na manutenção da homeostase metabólica, exercendo efeitos sobre o metabolismo glicêmico em diferentes níveis. Alterações nos níveis de hormônios tireoideanos, como ocorrem no hipertireoidismo e no hipotireoidismo, estão associados a aumento da resistência à insulina. A captação de glicose mediada pela insulina no músculo esquelético, um dos fatores determinantes da homeostase glicêmica, é dependente da expressão do transportador de glicose tipo 4 (GLUT-4) na membrana celular deste tecido. Visto que o gene do GLUT-4 é induzido pelos hormônios tireoideanos, alterações na concentração de triiodotironina (T3) tecidual podem explicar a associação entre alterações nos níveis de hormônios tireoideanos e resistência à insulina. No músculo esquelético a ativação do pro-hormônio tiroxina (T4) ao hormônio ativo T3 é regulada através da atividade da enzima desiodase tipo 2 (D2). Estudos em modelos animais nocaute para o gene da enzima D2 (D2K0) demonstraram resistência à insulina aumentada nestes animais. Em humanos, um polimorfismo da D2, no qual uma treonina (Tre) é trocada por uma alanina no códon 92 (D2 Tre92Ala), foi associado a redução da atividade da D2 em tecidos periféricos e maior resistência à insulina. O entendimento dos mecanismos que explicam a associação entre redução na atividade da D2 e resistência à insulina são de especial interesse, por suas potenciais implicações terapêuticas.

Introdução

Os hormônios tireoideanos têm importante papel na manutenção da homeostase metabólica, exercendo efeitos sobre o metabolismo glicêmico em diferentes níveis. A regulação da ativação dos hormônios tireoideanos nos tecidos periféricos, através da atividade das enzimas desiodases, ilustra o papel do metabolismo dos hormônios tireoideanos no controle da homeostase glicêmica.

Metabolismo dos hormônios da tireóide

Sob estimulo da tireotrofina (TSH) hipofisária, a glândula tireóide humana secreta diariamente cerca de 80-100µg do pró-hormônio tiroxina (T4) e 30-40 µg do hormônio ativo triiodotironina (T3). Em indivíduos eutireoideanos, aproximadamente 80-90% do hormônio T3 biologicamente ativo circulante deriva da conversão periférica de T4 em T3 (1).

A produção de T3 nos tecidos periféricos é regulada localmente através da ação das desiodases, um grupo de enzimas que representam uma etapa crítica na modulação pré-receptor da ação dos hormônios tireoideanos (2). As desiodases tipos 1, 2 e 3 (D1, D2 e D3) constituem uma família de oxiredutases que catalisam a remoção de uma molécula de iodo do anel externo (D1 e D2, ativação) e do anel interno (D3, inativação) dos hormônios tireoidianos (**Figura 1**)(2). A via da desiodação é um passo crítico na ativação e inativação do hormônio da tireóide, permitindo rápidas modificações no status tireoidiano intracelular de uma forma tecido-específica, sem afetar as concentrações circulantes dos mesmos. Assim, é possível controlar a concentração e a atividade intracelular de T3 independentemente dos níveis de T3 sérico.

A desiodação do T4 a T3 é necessária para ativar o principal produto secretado pela glândula tireóide. A D2 é uma desiodase exclusiva de anel externo que converte T4 em T3 e rT3 (T3 reverso) em T2. Ao contrário, a D1 pode promover a desiodação tanto do anel externo quanto do anel interno (**Figura 1**). Em humanos, os níveis mais altos de atividade da D1 são encontrados na tireóide, fígado e rim. A D2 é mais expressa na hipófise, cérebro, tireóide, pele, músculos esquelético e cardíaco (2). A D3 desempenha exclusivamente atividade de desiodação do anel interno, catalisando a conversão de T4 a rT3 e a conversão de T3 a 3,3'-T2, ambos produtos

biologicamente inativos (**Figura 1**). A D3 contribui para a homeostase do hormônio da tireóide protegendo os tecidos de um excesso de hormônio tireoidiano, sendo a principal enzima inativadora de T3 e T4. Estudos usando imunofluorescência com microscopia cofocal identificaram a D2 no retículo endoplasmático, enquanto a D1 e a D3 são localizadas na membrana plasmática (2).

Os hormônios tireoideanos circulantes são transportados através das membranas celulares, e o T3 que ganha acesso ao núcleo celular, interage com os receptores dos hormônios tireoideanos (THRα e THRβ, membros da família dos receptores nucleares celulares), induzindo e suprimindo a expressão de uma série de genes, alguns com importante papel no controle do metabolismo glicêmico (2).

Controle do metabolismo glicêmico

A homeostase glicêmica é determinada por mecanismos complexos, com contribuição distinta nos períodos de jejum e pós-prandial (3). No jejum, a produção endógena de glicose e a utilização de glicose pelos tecidos periféricos estão em equilíbrio. Neste período, o fígado responde por aproximadamente 85% da produção de glicose, com os 15% restantes provenientes do metabolismo renal, sendo cerca de 50% da produção endógena de glicose derivada de glicogenólise e 50% de gliconeogênese (3, 4).

No período pós-prandial, a absorção intestinal de glicose leva a aumento na glicemia, o que estimula a secreção de insulina pelas células β pancreáticas. Consequentemente, a hiperinsulinemia e a hiperglicemia resultantes suprimem a produção hepática de glicose e estimulam a captação de glicose pelo fígado e pelos tecidos periféricos (5). O músculo esquelético responde por cerca de 80% da glicose captada pelos tecidos periféricos neste período, e o tecido adiposo por aproximadamente 5% (5).

Portanto, no período de jejum, o fígado e no pós-prandial; o músculo esquelético e o tecido adiposo são os principais determinantes da homeostase glicêmica em indivíduos saudáveis. De fato, estes são os tecidos que determinam resistência a ação da insulina e a resultante hiperglicemia em indivíduos com diabetes tipo 2 (3). A resistência à insulina se manifesta pela

manutenção da produção de glicose hepática, mesmo na vigência de hiperglicemia e hiperinsulinemia, e pela redução na captação de glicose mediada pela insulina no músculo esquelético e no tecido adiposo (3).

O papel dos hormônios da tireóide no metabolismo glicêmico

Os hormônios da tireóide exercem efeito sobre o metabolismo glicêmico através de interações em diferentes níveis. A secreção de insulina pelas células β pancreáticas está reduzida no hipotireoidismo e aumentada no hipertireoidismo. Reconhecido como um estado de hiperglicemia reversível, no hipertireoidismo os hormônios da tireóide induzem a expressão hepática de uma série de genes envolvidos na gliconeogênese, o que explica o incremento na produção de glicose pelo fígado que acompanha o excesso de hormônios tireoideanos (6). De outra parte, no hipertireoidismo a captação de glicose mediada pela insulina, no músculo esquelético e no tecido adiposo, está aumentada (7, 8). A resistência à insulina e a hiperglicemia resultantes do excesso de hormônios tireoideanos circulantes ocorrem pelo fato de o aumento na produção de glicose hepática suplantar o incremento na captação de glicose mediada pela insulina nos tecidos periféricos.

De forma interessante, o hipotireoidismo também está associado a um estado de resistência à insulina, visto que a redução de hormônios tireoideanos circulantes leva a redução na captação de glicose mediada pela insulina no músculo esquelético (7). Devido a concomitantemente redução na produção hepática de glicose que ocorre no hipotireoidismo, a resistência à insulina nos tecidos periféricos pode não se manifestar com hiperglicemia.

A captação de glicose mediada pela insulina no músculo esquelético e no tecido adiposo é dependente da expressão do transportador de glicose tipo 4 (GLUT-4) na membrana celular destes tecidos. Visto que o gene do GLUT-4 contém em sua região promotora um elemento responsivo aos hormônios tireoideanos, e que estudos em modelos animais demonstraram que o tratamento com T3 aumenta a expressão do mRNA e da proteína do GLUT-4, pode-se supor que a redução da captação de glicose mediada pela insulina no hipotiroeidismo ocorre por menor expressão do GLUT-4 associado à condição (9).

O papel das desiodases na resistência à insulina

A enzima D2 desempenha papel crítico na manutenção dos níveis intracelulares de T3, controlando assim, de forma indireta, o metabolismo energético e a resposta dos tecidos periféricos à insulina (10). Devido a sua localização no reticulo endoplasmático, o T3 produzido pela D2 é especialmente efetivo em ganhar acesso ao núcleo e interagir com os receptores dos hormônios tireoideanos (1, 11). Genes de proteínas envolvidas no controle da homeostase glicêmica, como GLUT-4 e proteínas desacopladoras (UCPs), são controlados em nível transcripcional pelos hormônios tireoideanos. No tecido adiposo marrom (BAT), a expressão de UCPs aumenta a termogênese e o gasto energético, e no músculo esquelético, a expressão de GLUT-4 aumenta a captação de glicose pelo tecido (9, 12). Os efeitos positivos sobre a expressão de UCPs e GLUT-4 induzidos pelo T3 resultam na redução da resistência à insulina.

De fato, estudos com camundongos nocaute para o gene da D2 (D2K0) demonstraram que a não-expressão da enzima D2 leva a manifestação de um fenótipo de aumento da resistência à insulina e de tendência a ganho de peso nestes animais (12, 13). De forma interessante, os camundongos D2K0 manifestam fenótipo de aumento da resistência à insulina mesmo quando submetidos a dietas que não induzem alteração no peso, o que sugere uma associação direta entre diminuição da atividade da D2 e resistência à insulina (13).

Em humanos, sabe-se que polimorfismos nos genes das desiodases podem interferir na expressão destas enzimas, potencialmente alterando o metabolismo dos hormônios tireoideanos (1, 14-17). Um polimorfismo de nucleotídeo único (SNP) da D2, no qual uma treonina (Tre) é trocada por uma alanina no códon 92 (D2 Tre92Ala), foi associado com redução da atividade da D2 em tecidos periféricos e maior resistência à insulina em indivíduos não-diabéticos e em pacientes com diabetes tipo 2 (14, 18).

Acredita-se que no músculo esquelético de indivíduos com o genótipo Ala92Ala da D2 a geração de T3 intracelular pela D2 esteja diminuída, resultando em um estado de hipotireoidismo intracelular relativo, com conseqüente redução da expressão de genes induzidos pelo T3, como o GLUT-4 e UCPs, o que resulta em um fenótipo de aumento da resistência à

insulina. Entretanto, cabe ressaltar que estudos funcionais não identificaram modificações significativas nas propriedades bioquímicas da enzima mutante, o que sugere que esta variante parece ser um marcador de expressão anormal da D2, mas que a mutação *per se* não justifica a redução de atividade da enzima (14).

É interessante notar que o polimorfismo Tre92Ala da D2 também já foi associado a outras condições como aumento do risco para oestoartrose, hipetensão arterial sistêmica, Doença de Graves, alterações no quociente de inteligência associados a deficiência de iodo, bem-estar psicológico e resposta ao tratamento com T3 ou T4, redução da massa óssea e maior *turnover* ósseo (19-24). A maioria destas associações é independente dos níveis séricos de hormônios tireoideanos, o que ressalta a importância da regulação local do metabolismo dos hormônios tireoideanos nos tecidos periféricos.

Conclusão

Os hormônios tireoideanos exercem efeitos sobre o metabolismo glicêmico em diferentes níveis, e alterações no metabolismo dos hormônios tireoideanos estão associados a aumento da resistência à insulina. No músculo esquelético a captação de glicose mediada pela insulina é dependente da expressão do GLUT-4 na membrana celular deste tecido. Visto que o gene do GLUT-4 é induzido pelos hormônios tireoideanos, alterações na concentração de T3 tecidual através de modulação da atividade da enzima D2 podem explicar a associação entre alterações nos níveis teciduais de hormônios tireoideanos e resistência à insulina. Estudos em modelos animais D2K0 e do polimorfismo D2 Tre92Ala em humanos sugerem que a redução da atividade da D2 leva a um estado de hipotireoidismo intracelular relativo, com conseqüente redução da expressão de genes induzidos pelo T3, como o GLUT-4, o que resulta em um fenótipo de aumento da resistência à insulina.

O entendimento dos mecanismos moleculares que explicam a associação entre redução na atividade da D2 com aumento da resistência à insulina, sugere um papel central da regulação do T3 intracelular na fisiopatologia, e sinalizam o potencial de desenvolvimento de moléculas com alvo terapêutico específico.

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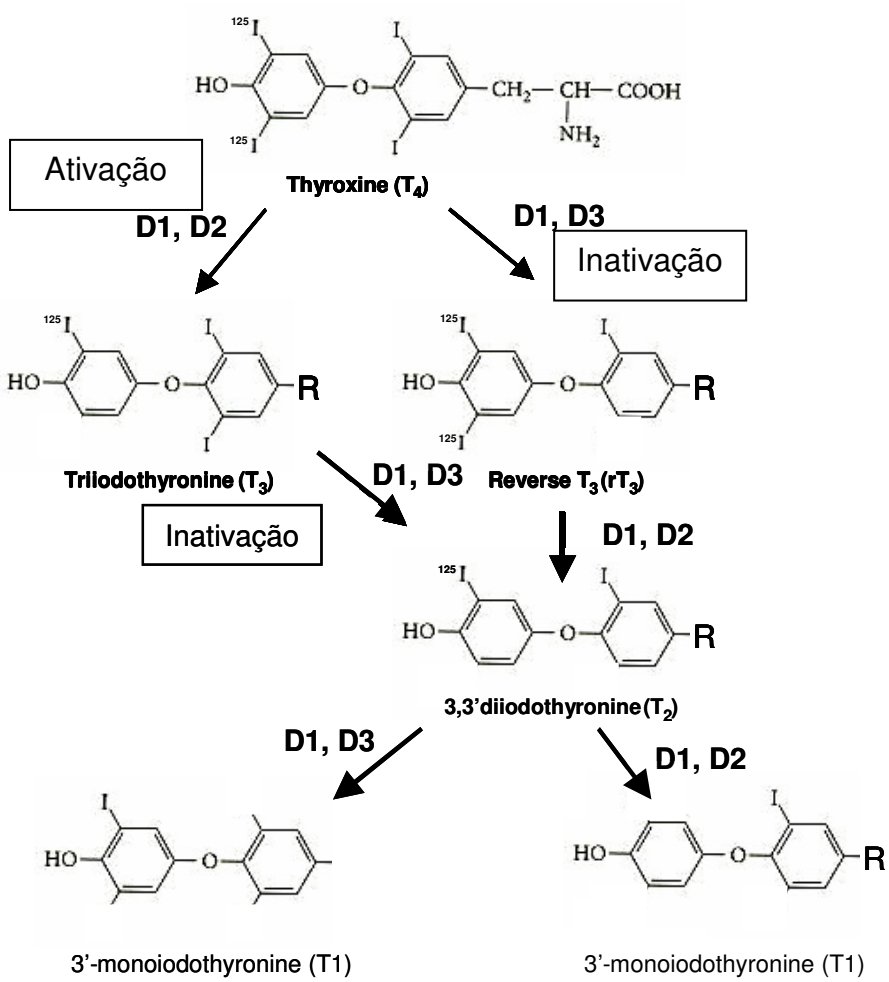

Figure 1. Metabolismo dos Hormônios Tireoidianos.

Association of the type 2 deiodinase Thr92Ala polymorphism with type 2 diabetes: case-control study and meta-analysis.

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Abstract

Objective: The type 2 deiodinase (D2) is a key enzyme for intracellular T3 generation. A single nucleotide polymorphism in D2 (Thr92Ala) has been associated with increased insulin resistance in non-diabetic and type 2 diabetes (DM2) subjects. Our aim was to evaluate whether the D2 Thr92Ala polymorphism is associated with increased risk for DM2. **Design and Methods**: A case-control study with 1057 DM2 and 516 non-diabetic subjects was performed. All participants underwent genotyping of the D2 Thr92Ala polymorphism. Additionally, systematic review and meta-analysis of the literature for genetic association studies of D2 Thr92Ala polymorphism and DM2 was performed in Medline, Embase, LiLacs, SciELO, and major meetings databases using the terms "rs225014" OR "thr92ala" OR "T92A" OR "dio2 a/g". **Results:** In the case-control study, the frequencies of D2 Ala92Ala homozygous were 16.4% (n=173) vs. 12.0% (n=62) in DM2 vs. controls, respectively, resulting in an adjusted odds ratio (OR) of 1.41 (CI95% 1.03-1.94, P=0.03). The literature search identified 3 studies that analyzed the association of the D2 Thr92Ala polymorphism with DM2, with the following effect estimates: Mentuccia (OR 1.40 [CI95% 0.78-2.51]), Grarup (OR 1.09 [CI95% 0.92-1.29]) and Maia (OR 1.22 [CI95% 0.78-1.92]). The pooled effect of the 4 studies resulted in an OR 1.18 (CI95% 1.03-1.36, P=0.02). **Conclusions**: Our results indicate that in a case-control study, the homozygosity for D2 Thr92Ala polymorphism is associated with increased risk for DM2. These results were confirmed by a meta-analysis including 11,033 individuals and support a role for intracellular T3 concentration in skeletal muscle on DM2 pathogenesis.

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Introduction

Thyroxine (T4), a major secretory product of the thyroid gland, needs to be converted to triiodothyronine (T3) to exert its biological activity. Type 2 deiodinase (D2) catalyzes T4 to T3 conversion, and plays a critical role in maintaining intracellular T3 levels in specialized tissues, such as the anterior pituitary, central nervous system, and brown adipose tissue (BAT) (1). D2 gene (*DIO2*) expression has also been reported in pituitary, thyroid, placenta, heart and skeletal muscle, and testis (2-8). Recently, **it has been suggested that D2 also contributes for a fraction of the serum T3 levels in euthyroid and hypothyroid individuals (9).**

Previous studies have demonstrated that polymorphisms in the deiodinase genes might interfere in the phenotypic expression of these enzymes (6, 10). Interestingly, a study described a single nucleotide polymorphism in D2, in which a threonine (Thr) change to alanine (Ala) at codon 92 (D2 Thr92Ala) was associated with an approximately 20% lower glucose disposal rate in non-diabetic subjects (11). In addition, the Ala allele in homozygosis was associated with greater insulin resistance in type 2 diabetes patients and decreased enzyme activity in human tissues (6). **The mechanism of reduced D2 activity, however, is still not clear since no significant changes in the biochemical properties of the mutant enzyme has been detected (12), thus suggesting that this variant could be only a marker for abnormal Dio2 expression.** The D2 Thr92Ala polymorphism has also been linked to increased risk for osteoarthritis (13), hypertension (14), Graves' disease (15), intelligence quotient alterations associated with iodine deficiency (16), psychological well-being and response to T3 or T4 treatment (17), and decreased bone mass and higher bone turnover (18). Intriguingly, most of these associations are independent of serum thyroid hormone levels, which highlight the importance of local regulation of thyroid hormones in peripheral tissues.

Type 2 diabetes is a heterogeneous group of disorders, with varying degrees of insulin insufficiency and insulin resistance, which result in increased blood glucose concentrations. At last, insulin resistance results either from inappropriately increased hepatic gluconeogenesis and/or decreased glucose disposal rate in tissues such as skeletal muscle and adipose tissue. Glucose transporter type 4 (GLUT4), the insulin-responsive glucose transporter, mediates the rate-limiting step of glucose metabolism. Thyroid hormones are known to up-regulate the expression of GLUT4 in skeletal muscle, and consequently increase glucose uptake (19). Thus, one could speculate that a lower intracellular D2-generated T3 in skeletal muscle could create a state of relative intracellular hypothyroidism, decreasing the expression of genes involved in energy use, such as GLUT4, resulting in increased insulin resistance. Nevertheless, population-based studies failed to demonstrate an association between the D2 Thr92Ala polymorphism and increased risk for type 2 diabetes (20-22).

Clinical and experimental data support a biological plausibility for a role of the D2 Thr92Ala variant in predisposition to type 2 diabetes, a heterogeneous disease with many environmental and genetic factors interactions. In this setting, where both environment factors and multiple genes play a role in pathophysiology, it is not unexpected that genetic association studies fail to show an association, even when it actually exists. Accordingly, diabetes has been called “a geneticist’s nightmare” and, in this context, a huge number of patients may be needed to clarify the collaboration of a single polymorphism for this polygenic disease (23). Here, we sought to further test the hypothesis that homozygosity for the D2 Thr92Ala polymorphism is associated with increased risk for type 2 diabetes. In an attempt to address the study limitations highlighted above, we performed a case-control study in a Brazilian population and a meta-analysis of the literature on the subject.

Research Design and Methods

Case-Control Study

Type 2 diabetes Population

The sample population consists of 1057 type 2 diabetes patients participating in a multicenter study that started recruiting patients in Southern Brazil in 2002. That study aimed to evaluate risk factors for type 2 diabetes and its complications. Initially, it included four centres located at general hospitals in the State of Rio Grande do Sul, namely Grupo Hospitalar Conceição, Hospital São Vicente de Paula, Hospital Universitário de Rio Grande, and Hospital de Clínicas de Porto Alegre. The detailed description of that study can be found elsewhere (24). **The sample population presented here includes a subgroup of 183 patients described in a previous study (6).**

All patients were of European ancestry (mostly descendants of Portuguese, Spanish, Italians and Germans). The ethnic group was defined on the basis of self-classification and subjective classification (skin color, nose and lip shapes, hair texture, and family history). A standard questionnaire was used to collect information about age, age at type 2 diabetes diagnosis, and drug treatment. All patients underwent physical and laboratory evaluations. They were weighed without shoes and in light outdoor clothes and had their height measured. Body mass index (BMI) was calculated as weight (kg) / height (meters)^2. Blood pressure (BP) was measured twice after a 5-min rest in the sitting position using a mercury sphygmomanometer (Korotkoff phases I and V). The mean value of two measurements was used to calculate systolic and diastolic BP.

Diabetes was defined as treatment with either insulin or an oral hypoglycemic agent or a fasting plasma glucose of at least 126 mg/dL (7.0 mmol/L) at two or more examinations, 2-hour 75g oral glucose tolerance test plasma glucose of at least 200

mg/dL (11.1 mmol/L), or random plasma glucose of 200 mg/dL (11.1 mmol/L) or higher (25). Patients were classified as type 2 diabetes based on patients' age (30 year old or older), upon the need or not for insulin at diagnosis, and absence of ketones in the urine. Micro and macrovascular complications were accessed at study entry.

Non- diabetic Control Population

A group of 516 non-diabetic **volunteers** attending the blood-donation facility of Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil) constituted our control group. A standard questionnaire was used to collect information about age, sex, skin color, and presence of comorbidities (eg. systemic arterial hypertension) and drug treatment from controls.

Protocol Ethical Approval

The information obtained from the study did not influence the patient's diagnosis or treatment. The local Ethics Committee approved the protocol, and all patients signed an informed consent form.

Laboratory Tests

In type 2 diabetes mellitus patients, a serum sample was collected after a 12-h fast. Glucose levels were determined by a glucose oxidase method and glycated hemoglobin (A1c) by an ion-exchange HPLC procedure (Merck-Hitachi L-9100 glycated hemoglobin analyzer, Merck, Darmstadt, Germany; reference range: 2.7–4.3%). Serum insulin was measured by **electrochemiluminescence** (ElecsysR Systems 1010/2010/modular analytics E170, Roche Diagnostics, Indianapolis, IN). **The intra-assay and inter-assay coefficient of variation were 1.5% and 4.9%, respectively.** Insulin sensitivity was estimated by homeostasis model

assessment [HOMA = fasting insulin (milliunits per milliliter) x fasting glucose (millimoles per liter)/22.5], as recently described and validated (26). Additionally, triglyceride and cholesterol levels were measured by enzymatic methods and low-density lipoprotein-cholesterol calculated using the Friedewald equation (LDL cholesterol = total cholesterol – HDL cholesterol – triglycerides/5).

In non-diabetic subjects no laboratory measures were performed.

Genotyping

DNA was extracted from peripheral blood leukocytes by a standardized salting-out procedure. Primers and probes contained in the Human Custom TaqMan Genotyping Assay 40x (Applied Biosystems, Foster City, CA; USA) were used for genotyping our samples. One allelic probe was labeled with VIC dye and the other was labeled with FAM dye. The reactions were conducted in a 96-well plate, in a total 5 µl reaction volume using 2ng of genomic DNA, TaqMan Genotyping Master Mix 1x (Applied Biosystems), and Custom TaqMan Genotyping Assay 1x. The plates were then positioned in a real-time PCR thermal cycler (7500 Fast Real PCR System; Applied Biosystems) and heated for 10 minutes at 95° C followed by 50 cycles of 95℃ for 15 seconds and 63℃ for 1 minute. Fluorescence data files from each plate were analyzed using automated allele-calling software (SDS 2.1; Applied Biosystems).

Patients were classified in groups of Ala/Ala or Ala/Thr-Thr/Thr according to the presence of the Ala allele. All amplification reactions were performed twice. The genotyping success was more than 95%, with a calculated error rate based on PCR duplicates of 0%.

Statistical Analyses

Results are expressed as frequencies, mean ± standard deviation (27) or median and percentile 25-75 (P25-75). Allelic frequencies were determined by gene counting, and departures from the Hardy-Weinberg equilibrium were verified using χ^2 tests. Clinical and laboratory data were compared using χ^2, unpaired Student's t test, Mann-Whitney U test, ANOVA, Kruskal-Wallis H test or multiple logistic regression analysis as appropriate. A two-tailed $P < 0.05$ was considered statistically significant, and all analyses were performed by SPSS version 15.0 (SPSS, Chicago, IL, USA).

Meta-Analysis

Search Strategy

The electronic databases Medline, Embase, LiLacs and SciELO were searched for studies of genetic association between the D2 Thr92Ala polymorphism and type 2 diabetes. We also searched the abstracts of the major diabetes and thyroid meetings over the last four years. We limited the search to humans and used the following strategy: "rs225014" OR "thr92ala" OR "T92A" OR "dio2 a/g". The reference lists of all identified articles were also searched, and authors of included studies were consulted to obtain additional information when needed. For inclusion in the meta-analysis, we considered as attending the inclusion criteria both: 1) observational studies (cohort, case-control and cross-sectional studies) on the D2 Thr92Ala polymorphism; 2) that included patients with and without type 2 diabetes.

Two investigators (JMD and WEM), blinded to each other's rating, independently assessed study eligibility. All data were independently abstracted in duplicate using a standardized abstraction form. Differences in data extraction were resolved by a third party (ALM) and by referencing the original publication.

Statistical Analyses

Data from the selected studies were retrieved and annotated according to the presence or absence of type 2 diabetes. The frequencies of each genotype of the D2 Thr92Ala polymorphism, in type 2 diabetes and in non-diabetic controls from all studies were pooled. The odds ratios (OR) and their 95% confidence intervals (CI95%) for individual studies, and for the pooled effect were calculated with the Mantel-Haenszel, the DerSimonian and Laird, and the Peto's methods, using random-effects and fixed-effects models. Heterogeneity was tested with the Cochran Q test and inconsistency accessed through the I^2. Additionally, sensitivity analysis was performed omitting one study at a time to evaluate the influence of each study on the pooled estimate. We used the programs Review Manager 5 (28) and MIX version 1.7 (29) for data analysis, and the study was designed and described in accordance with current guidelines (30-32).

Results

Case-Control Study

The baseline characteristics of the 1057 type 2 diabetes patients and 516 non-diabetic control subjects regarding age in years and sex were, respectively, the following: mean age 59.3 ± 10.0 (age at diagnosis 47.4 ± 10.9) and 46.2 ± 8.8, P=0.02; females comprised 53% (n = 558) and 37% (n = 191) of study groups, P<0.005 (**Table 1**).

The frequency of the minor Ala allele was 0.38 in type 2 diabetes patients and control subjects. In the type 2 diabetes group, 381 (36%) individuals were homozygous for the Thr allele, 503 (47.6%) were heterozygous (Thr/Ala), and 173 (16.4%) were homozygous for the Ala allele. In the group control, 195 subjects for the Thr allele (37.8%), 259 (50.2%) were heterozygous and 62 (12.0%) were homozygous for Ala allele. The genotypes were in Hardy-Weinberg equilibrium (P=0.96). The frequency of homozygotes for the Ala allele was significantly higher in the type 2 diabetes group

than in controls (16.4% vs. 12.0%, respectively; P=0.03). This resulted in an OR of 1.43 (CI95% 1.05-1.96, P=0.03) for the Ala/Ala genotype in type 2 diabetes patients. Because the type 2 diabetes and control groups differed by age and sex (Table 1), we performed a multiple logistic regression analysis with age, sex, and genotype as independent variables and type 2 diabetes as the dependent variable. The Ala/Ala genotype remained significantly associated with type 2 diabetes with an adjusted OR of 1.41 (CI95% 1.03-1.94, P=0.03).

Assuming a recessive model, patients with Ala/Thr and Thr/Thr genotypes were grouped and compared with patients with Ala/Ala genotype. The diabetes duration (10 [5-17] years), systolic (142 ± 23 mmHg) and diastolic (85 ± 13 mmHg) BP, microvascular (67%) and macrovascular (58%) complications, renal function (creatinine 0.9 [0.8-1.1] mg/dL) and non-glycemic metabolic control (HDL cholesterol 45 ± 12 mg/dL; LDL cholesterol 129 ± 42 mg/dL; total cholesterol 208 ± 46 mg/dL and triglycerides 150 [105-217] mg/dL) were similar across the D2 genotypes in the type 2 diabetes patients (data not shown). There was a tendency towards higher fasting glucose levels in the type 2 diabetes patients harboring the Ala/Ala genotype (fasting glucose 173 ± 71 mg/dL vs. 167 ± 63 mg/dL, for Ala/Ala vs. Ala/Thr-Thr/Thr, respectively, P=0.06). Confirming our previous report, subjects with the Ala/Ala genotype had increased insulin levels (fasting insulin 16.8 [8.9-25.8] mIU/mL vs. 11.3 [6.7-18.1] mIU/mL, for Ala/Ala vs. Ala/Thr-Thr/Thr, respectively, P=0.01) and increased insulin resistance (HOMA index 8.5 [5.2-14.1] vs. 4.5 [2.5-8.1], for Ala/Ala vs. Ala/Thr-Thr/Thr, respectively, P<0.005). Moreover, the glicemic control, assessed by A1c levels, were worst in the group of patients with the Ala/Ala genotype (A1c 8.4 ± 2.6 % vs. 7.6 ± 2.5 %, respectively, P=004), despite comparable anti-diabetic therapy (metformin 42.7% vs. 37.7%, p=0.44; sulfonylureas 34.7% vs. 31.7, p=0.60; insulin 41.4% vs. 40.2%, p=0.82 for Ala/Ala vs. Ala/Thr-Thr/Thr, respectively). **Eighty-four patients (8%) were not receiving drug therapy (diet / exercise alone).**

Meta-analysis

A literature search using the terms "rs225014" OR "thr92ala" OR "T92A" OR "dio2 a/g", retrieved 28 articles dealing with D2 Thr92Ala polymorphism. Out of them, 18 studies were excluded because they did not include data on the presence or absence of diabetes, 4 because the presence of diabetes was an exclusion criteria, 1 because it included only diabetic patients and 1 because data of the diabetic and non-diabetic populations was non-extractable. Thus, we identified 3 observational studies (2 cross-sectional and 1 case-control) that analyzed the Thr92Ala polymorphism in type 2 diabetes and non-diabetic subjects (**Figure 1**) (20-22). Therefore, the meta-analysis included 4 studies: the 3 identified through database search, and our case-control study. The demographic and glycemic characteristics of the populations included in each study are described in **Table 2**.

The study of Mentuccia et al.(20) looked at the Amish population (OR 1.40, IC95% 0.78-2.51), Maia et al.(21) analyzed data from the Framingham Study (OR 1.22, IC95% 0.78-1.92); and Grarup et al.(22) studied a large cohort of Danish (OR 1.09, IC95% 0.92-1.29). Neither heterogeneity (Q=2.64, p=0.45) nor inconsistency (I^2=0%) across studies were detected. Combining the results of the 4 studies, applying the fixed-effects model resulted in an estimate Peto pooled OR of 1.18 (CI95% 1.03-1.36, p=0.02) (**Figure 2**). The results were very similar (all statistically significant), when using the Mantel-Haenszel, the DerSimonian and Laird methods, and the random-effects model (data not shown).

The weight of each study on the pooled estimate was as follows: Mentuccia 5.6%, Maia 9.3%, Grarup 65.3% and ours 19.7%. Omission of one study at a time was performed to verify the impact of each study on the estimate effect. Analysis with the omission of the studies of Mentuccia, Maia, Grarup and ours resulted in similar effect estimates, with calculated OR of 1.18 (CI95% 1.00-1.40, change in OR estimate +0.4%), 1.21 (CI95% 0.99-1.47, change in OR estimate +2.8%), 1.37 (CI95% 1.08-1.73, change in OR estimate +16.2%), and 1.12 (CI95% 0.96-1.31, change in OR

estimate -4.7%), respectively. All the calculations of the sensitivity analysis provided effect estimates similar to the pooled OR of the 4 studies, reinforcing the homogeneity between the studies included in our meta-analysis, and excluding a dominant influence of one study in the magnitude of the effect estimate.

Discussion

Type 2 diabetes is a highly heterogeneous disease with multiple environmental and genetic factors involved in its pathogenesis. Here, we have performed a case-control study and a meta-analysis of genetic association studies which demonstrate that homozygosis for the Ala allele of the single nucleotide polymorphism Thr/Ala in codon 92 of the D2 is associated with increased risk for type 2 diabetes in the general population.

D2 is a key enzyme in determining intracellular T3 concentration and might have a critical role in metabolic activity of skeletal muscle, analogous to its role in BAT (1, 33, 34). Chronic adrenergic stimulation in adult humans was found to increase both resting energy expenditure (35) and serum T3 to T4 ratio (33), suggesting the existence of an adrenergic dependent T4 to T3 conversion pathway (36). In addition, in patients receiving T4 replacement, resting energy expenditure correlated directly with free T4 and inversely with serum TSH but, interestingly, not with serum T3 (34). These data are consistent with a role for T4 via D2-dependent intracellular T3 production in skeletal muscle as a significant physiological determinant of energy expenditure in humans. Moreover, recent evidence shows that BAT is present and active in adult humans, and that D2-mediated T3 production in this tissue might be important for thermal adaptation and metabolic activity (37).

Previous studies demonstrated that homozygosity for the Ala allele of the D2 Thr92Ala polymorphism was associated with an approximately 20% lower glucose disposal rate in non-diabetic Caucasians (11). The frequency of the variant allele was also found to be increased in some ethnic groups, such as Pima Indians and Mexican-

Americans, who also have a higher prevalence of insulin resistance (11). Accordingly, we have previously demonstrated that the D2 Ala/Ala genotype was associated with increased insulin levels and HOMA index in patients with type 2 diabetes whereas decreased D2 activity was found in sample biopsies of individuals harboring this genotype (7). Here, we have further confirmed the D2 Ala/Ala genotype association with increased insulin resistance (increased HOMA index), and demonstrated that this genotype is also associated with worse glycemic control (increased A1c levels) in a cohort of 1057 type 2 diabetes subjects.

Taken together, these observations raised the hypothesis that the D2 Thr92Ala polymorphism would be associated with increased risk for type 2 diabetes. However, despite a statistically non-significant tendency, all studies performed on this subject have failed to demonstrate such an association (**Figure 2**). Using cross-sectional designs, Mentuccia et al.(20) have studied 1,268 subjects of the Old Order of Amish (Pennsylvania, USA) whereas Maia et al.(21) evaluated a subset of 1,631 subjects from the Offspring Cohort of the Framingham Heart Study (Massachusetts, USA). Type 2 diabetes comprised 14.1% (179 individuals) and 10.4% (170 individuals) of the populations of Mentuccia and Maia, respectively.

Grarup et al.(22) studied 7,342 white subjects from Glostrup and Copenhagen (Denmark), in a mixed case-control and cross-sectional design. In this study, in the unadjusted analyses, an association was verified between the D2 Ala/Ala genotype and glycemic traits of insulin resistance: an increased area under serum insulin curve during the oral glucose tolerance test and elevated fasting plasma glucose in the D2 Ala/Ala group. No increased risk for type 2 diabetes was found. It is interesting to highlight, however, that the control group of this study was comprised of patients younger than the cases (age of 46.4 ± 8.8 vs. 51.3 ± 11.2 years, for controls vs. type 2 diabetes patients at diagnosis, respectively; difference 4.9 years [CI95% 4.3-5.5], $p<0.001$).

The contribution of a single gene to a polygenic disease is determined by the prevalence of the implicated allele and the magnitude of the association with the condition (38). In this context, underpowerment is a concern in genetic association studies in a disease such as type 2 diabetes. For instance, despite all the research efforts on this area, the type 2 diabetes has only a 6% estimated proportion of heritability explained by the 19 loci associated with the disease (27, 38). The magnitude of effect of the described genetic variants associated to the disease are of OR 1.14; 1.12-1.16 (median; P25-75) (23). In this scenario, the assumptions made in previous studies (21, 22) for a presumed magnitude of a Thr92Ala polymorphism association with type 2 diabetes of OR 1.9 and 1.3 might be over estimated.

Therefore, assuming a smaller magnitude of effect for the D2 Thr92Ala polymorphism on type 2 diabetes risk and the need of a large number of patients to rule out a role of this gene, we have designed a case-control study that included a large number of type 2 diabetic patients (1057 individuals) and performed a meta-analysis of the data on the subject. In the case-control study, the frequency of the Ala allele in homozygosis was significantly higher in type 2 diabetes than in control subjects (16.4 vs.12.0%, P=0.03). These frequencies, after adjusted for sex and age, resulted in an OR of 1.41 (CI95% 1.03-1.94) for Ala/Ala genotype in type 2 diabetes patients. The literature search identified 1 case-control and 2 cross-sectional studies on the subject, detailed above (Table 2). Despite the different designs employed (case-controls and cross-sectionals) and the different genetic background of the populations (North-Americans, Europeans and South-Americans), the results showed neither inconsistency nor heterogeneity in the pooled data from the 4 studies. The direction of the estimate was the same in the 4 studies, and moreover, the sensitivity analysis demonstrated that the effect estimate was consistent across studies. The meta-analysis OR of 1.18 (CI95% 1.03-1.35), reflects an increased risk for type 2 diabetes attributable to the homozigozity of the Ala allele of around 10% (CI95% 1-21%). This is a meaningful magnitude of effect for a single polymorphism in a polygenic disease like

type 2 diabetes. As an example, the Ala allele of the well characterized Pro12Ala substitution in the peroxisome proliferator-activated receptor 2 (PPARγ) gene accounts for a near 20% decreased risk for type 2 diabetes (39), and most of the other genes implicated in type 2 diabetes risk have magnitudes of effects less than 10% (23).

The finding that D2 single nucleotide polymorphism is associated with increased risk for type 2 diabetes has relevant clinical implications. One notable aspect is that most of the genetic loci that were identified in association studies of type 2 diabetes seem to affect insulin secretion (23), not insulin sensitivity such is this case. In the context of the current pandemics of obesity and obesity-attributable insulin resistance, the identification of a pathogenic genetic trait that contributes to increased insulin resistance and increased risk for type 2 diabetes constitutes an important step for better understanding of the mechanism of disease. Indeed, these results might support a role for intracellular T3 concentration in type 2 diabetes pathogenesis and might constitute a potential target to specific therapies (1, 7, 9).

Despite of all our efforts, we are aware that some factors unrelated to the D2 Thr92Ala polymorphism could have interfered with the findings of this study. Meta-analysis method is notoriously prone to publication bias, and although we have attempted to trace unpublished observations, we cannot assure that small negative studies were overlooked. Moreover, one of the identified studies was not included in this meta-analysis because data regarding diabetic population was unextractable (40).

In conclusion, our results demonstrate that the Ala/Ala genotype of D2 is associated with increased risk for type 2 diabetes. In a case-control study of 1,573 patients and in a meta-analysis of 4 studies with 11,033 subjects, this genotype was associated with increased risk for type 2 diabetes in the general population, a finding that might represent an advance thought understanding the genetic contribution to the pathogenesis of the disease.

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Table 1. Characteristics of type 2 diabetes (all and according to the D2 genotype) and non-diabetic individuals from our case-control study.

	Type 2 diabetes			Non-diabetics
	All (n = 1057)	Ala/Ala (n = 173)	Ala/Thr-Thr/Thr (n= 884)	All (n=516)
Age (years)[*a]	47.4 ± 10.9	46.9 ± 10.7	47.5 ± 11.0	46.2 ± 8.8
Female sex [n (%)][a]	558 (52.8)	98 (56.6)	460 (52.0)	191 (37.1)
Fasting glucose (mg/dL)	169 ± 65	173.3 ± 71.6	167.6 ± 63.3	NA
Fasting insulin (mIU/mL)[b]	11.7 (6.8-18.7)	16.8 (8.9-25.8)	11.3 (6.7-18.1)	NA
A1c (%)[b]	7.7 ± 2.5	8.4 ± 2.6	7.6 ± 2.5	NA
HOMA index[b]	5.1 (2.6-8.8)	8.5 (5.2-14.1)	4.5 (2.5-8.1)	NA
A allele frequency	0.38			0.38

Data are expressed as mean ± SD or median (P25-P75). NA, not available; A1c, glycated hemoglobin; HOMA, homeostasis model assessment [fasting insulin (mIU/mL) x fasting glucose (mmols/L)/22.5]. To convert glucose from mg/dL to mmol/L, divide by 18 or multiply by 0.055.

[*] For type 2 diabetes age at diagnosis was used for comparison with age of controls.
[a] Statistically significant comparisons between all type 2 diabetes vs. non-diabetic subjects (P=0.02 for age and P<0.005 for sex)
[b] Statistically significant comparisons between Ala/Ala vs. Ala/Thr-Thr/Thr genotypes (P=0.01 for fasting insulin, P=0.01 for A1c and P<0.005 for HOMA index)
Statistical analysis: unpaired Student's t test for age, fasting glucose and A1c; Mann-Whitney U test for fasting insulin and HOMA; and χ^2, for sex and A allele frequency.

Table 2. Characteristics of the populations of the 4 studies that evaluated the genetic association of the D2 Thr92Ala polymorphism with type 2 diabetes.

Author (reference)	Mentuccia (20)	Maia (21)	Grarup (22)	Dora
Year of publication	2005	2007	2007	2010
Individuals studied (n)	1268	1631	7000	1573
Type 2 diabetes subjects [n (%)]	179 (14.1)	170 (10.4)	1405 (20.1)	1057 (67.2)
Age (years)	45.5 ± 0.6	62.0 ± 9.0	48.5 ± 9.2	54.8 ± 11.4
Female sex – [n (%)]	707 (55.8)	841 (51.6)	3563 (50.9)	750 (47.7)
BMI (kg/m^2)	27.2 ± 0.2	28.2 ± 5.2	29.6 ± 5.3*	28.9 ± 5.0*
Fasting glucose (mg/dl)	91 ± 1	106 ± 27	NA	169 ± 65*
A1c (%)	5.2 ± 0.1	5.7 ± 1.0	7.8 ± 1.7*	7.7 ± 2.5*
A allele frequency	0.30	0.37	0.36	0.38

Data are expressed as mean ± SD. BMI, body mass index [weight (kg) / height $(meters)^2$]; NA, not available; A1c, glycated hemoglobin. To convert glucose from mg/dL to mmol/L, divide by 18 or multiply by 0.055.
*Data from the type 2 diabetes subjects of the study.

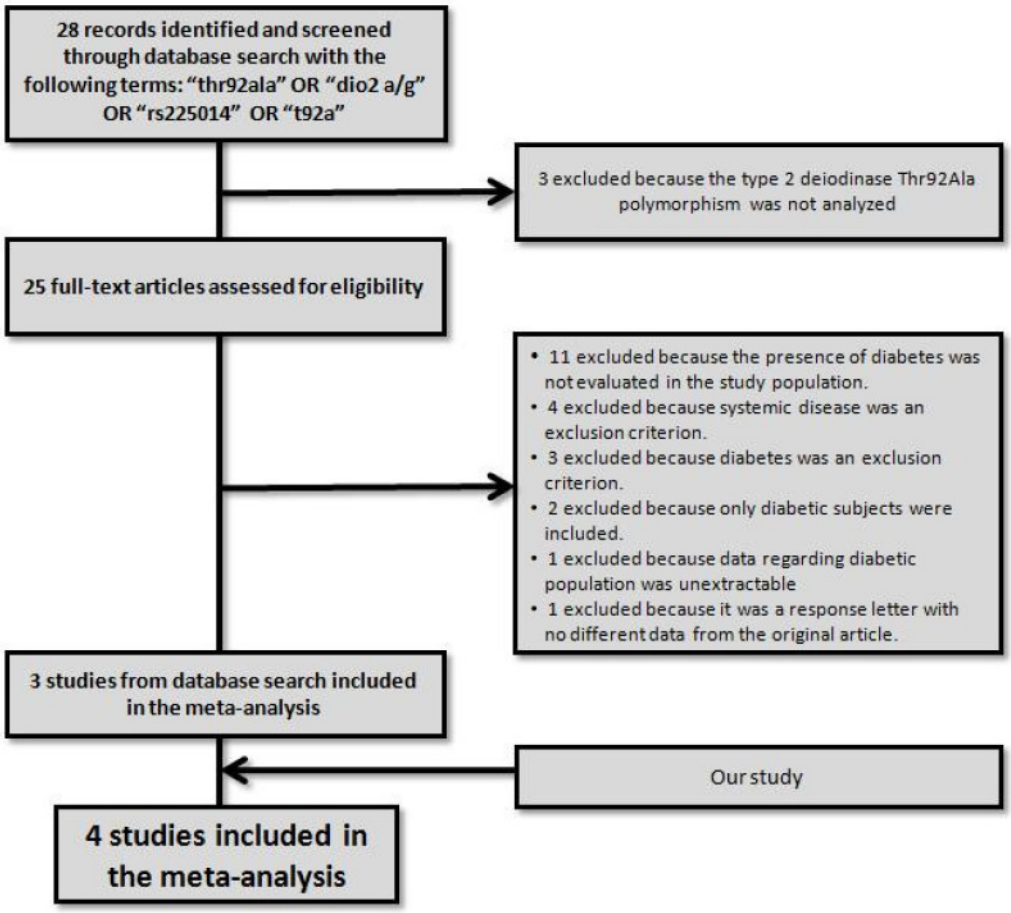

Figure 1. Flowchart of search results for the meta-analysis of the D2 Thr92Ala polymorphism association with type 2 diabetes.

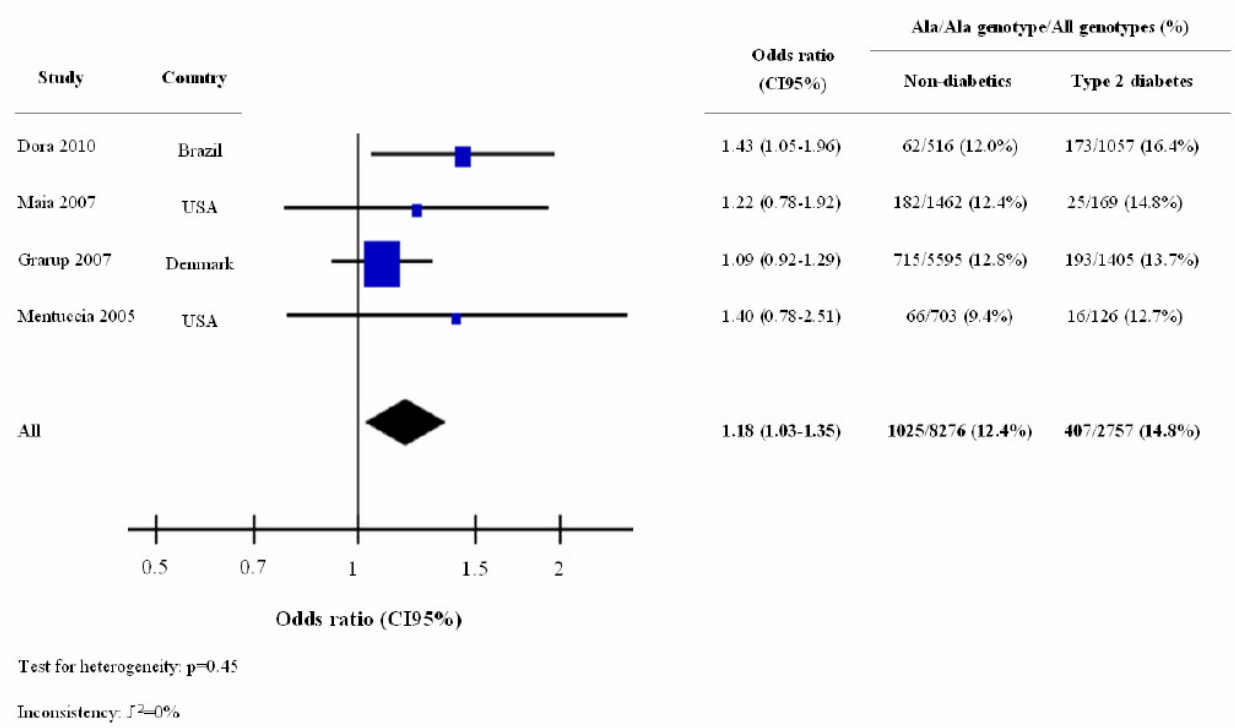

Figure 2. Individual and pooled odds ratios (OR) and 95% confidence intervals (CI95%) estimates for type 2 diabetes association to the Ala/Ala genotype of the type 2 deiodinase.

Statistical analysis: pooled OR estimated through the Peto method (fixed-effects model); heterogeneity tested with the Cochran Q test (Q=2.64, p=0.45) and inconsistency accessed through the I^2 (I^2=0%).