Thyroid hormone action is an important determinant of energy and glucose metabolism. T4 metabolism is regulated by the deiodinases of which type 2 is expressed in humans in skeletal muscle and brown adipose tissue, where its transcription is stimulated by the β3-adrenergic pathway. We performed molecular scanning of the human type 2 deiodinase (DIO2) gene and evaluated a novel variant for associations with obesity and insulin resistance, assessing both the main effect and interaction with the Trp64Arg ADRB3 variant. Molecular scanning of DIO2 in 50 obese Caucasians demonstrated a Thr92Ala variant. Association studies in 972 nondiabetic patients, 135 of whom underwent euglycemic-hyperinsulinemic clamps, showed that subjects with the Thr92Ala variant had lower glucose disposal rate (0.54 ± 0.02 mg · min⁻¹ · kg⁻¹ fat-free mass Ala92 homozygotes vs. 0.44 ± 0.02 Ala92 heterozygotes vs. 0.42 ± 0.04 Thr92 homozygotes, P = 0.0088). Association analysis of the entire group showed significant evidence for a synergistic effect between the Thr92Ala DIO2 and Trp64Arg ADRB3 variants on BMI (both variants 34.3 ± 0.9 kg/m² vs. neither variant 33.1 ± 0.4 kg/m², P = 0.04 for interaction). To our knowledge, Thr92Ala is the first description of a missense mutation of DIO2. This variant strongly associates with insulin resistance and, in subjects with the Trp64Arg ADRB3 variant, an increased BMI, suggesting an interaction between these two common gene variants. Diabetes 51:880–883, 2002

The cluster of obesity, hypertension, insulin resistance, and glucose intolerance/diabetes (Syndrome X) is a polygenic multifactorial condition in which the phenotype is the net result of the interaction between environmental factors and genetic predisposition deriving from variations in genes encoding proteins involved in metabolic pathways (1). In fact, polymorphisms in several candidate genes, such as the β3-adrenergic receptor (ADRB3) (2,3), insulin receptor substrate-1 (4), peroxisome proliferator–activated receptor-γ (5), fatty acid binding protein-2 (6), and perhaps others, have shown association with various traits of Syndrome X.

Thyroid hormones are important elements in the regulation of metabolic rate (7,8) and, although the molecular and cell biology of this process is not yet fully understood, evidence suggests that the thyroid hormones stimulate resting metabolic rate (RMR) by increasing ATP expenditure and through the regulation of expression of uncoupling proteins in the mitochondria of fat and muscle (9). Thyroid hormone also modulates adrenergic receptor number and thus responsiveness to catecholamines, which are also regulators of metabolic rate (10). Thus, thyroid hormone action is an extremely important determinant of the maintenance of the energy homeostasis. Furthermore, thyroid hormone influences carbohydrate metabolism in skeletal muscle and adipose tissue via the positive transcriptional regulation of the muscle/fat specific GLUT4 (11,12).

The deiodinases play a key role in the maintenance of circulating and tissue levels of thyroid hormones. The pro-hormone T4 is converted in the periphery to its active form, T3, or to its inactive metabolite, reverse T3, by the action of these enzymes (13). The deiodinases are selenoenzymes characterized by a selenocysteine in the catalytic domain of the enzyme encoded by a UGA codon in the presence of a characteristic 3’ untranslated region stem loop structure, the selenocysteine insertion sequence (SECIS) (14). Type 2 deiodinase appears to be a tissue-specific regulator of the intracellular T3 concentrations in brown fat, brain, and pituitary. In humans, type 2 deiodinase is
also expressed in skeletal muscle (15). The type 2 deiodinase gene (DIO2) is localized on chromosome 14q24.3, and the entire gene is encoded by three exons (16). Experimental evidence demonstrates that DIO2 activity is regulated by metabolic stressors, such as cold exposure and adrenergic stimulation, via the generation of intracellular cAMP (17,18). Furthermore, the action of this enzyme provides the supply of T3 for local use by the tissue itself, thereby creating a type of autoregulatory, autocrine feedback loop (19). We thus decided to study DIO2 as a candidate gene for obesity and insulin resistance in a population of morbidly obese Caucasians with normal thyroid function and to perform association studies of the gene variants in a well-characterized nondiabetic Caucasian population (Table 1).

By performing gene scanning of the entire coding region and the SECIS element of DIO2 with PCR–single strand conformation polymorphism (SSCP) analysis, followed by DNA sequence analysis, we identified a common nonconservative variant, 274 A→G, that predicts a nonconservative Thr92Ala substitution (Fig. 1). No other variants were observed. The allele frequency of the Thr92Ala DIO2 variant was 0.35 in the study population with a distribution meeting Hardy-Weinberg equilibrium. This variant was common in various ethnic groups, particularly in Pima Indians and Mexican-Americans with allele frequencies of 0.75 and 0.42, respectively (see online appendix at http://diabetes.diabetesjournals.org).

Association studies were performed in a large, well-characterized nondiabetic Caucasian population (20) recruited for energy balance studies. Hyperinsulinemic-euglycemic clamp studies were performed in a subgroup of 135 nondiabetic women. In this subgroup, there were no significant differences between genotypes in age, weight, BMI, fasting glucose levels, or RMR (Table 2). However, we observed a marked decrease in the glucose disposal rate in subjects with the Thr92Ala variant (Ala92 homozygotes 17.2 ± 1.6 mmol/min vs. Ala92 heterozygotes 18.2 ± 0.8 mmol/min vs. Thr92 homozygotes 22.6 ± 0.9 mmol/min, \( P = 0.0006 \), consistent with a dominant model (Table 2).

A trend toward an increase of fasting insulin level was observed in carriers of the Ala92 DIO2 allele (Ala92 homozygotes 105.0 ± 18.6 pmol/l vs. heterozygotes 72.0 ± 9.6 pmol/l vs. Thr92 homozygotes 57.0 ± 10.2 pmol/l, \( P = 0.0814 \) consistent with greater insulin resistance. In the larger group (\( n = 922 \)), the Thr92Ala DIO2 polymorphism did not associate with body weight or BMI. However, significantly higher weight and BMI were observed in carriers of both the Thr92Ala DIO2 and Trp64Arg ADRB3 variants compared with subjects with neither or either variant alone, suggesting an interaction between the two (Table 3).

To our knowledge, this is the first description of a missense variant of the human DIO2 gene. Although the crystal structure of the type 2 deiodinase is not yet known, it is worth noting that this nonconservative amino acid change (aliphatic for polar group), which is not located within the conserved deiodinase catalytic domain, could potentially affect its activity (21). However, this region of the enzyme is not phylogenetically conserved. The homologous amino acid is represented by a proline in rodents and by a glycine in chick. By contrast, humans and amphibians share a threonine in this position. The functional consequences of this common missense mutation are not yet known.

Our results indicate that the DIO2 Thr92Ala variant strongly associates with insulin resistance as measured by the hyperinsulinemic-euglycemic clamp. An inactivating mutation in DIO2 could lead to decreased intracellular availability of active thyroid hormone. A reduction in T3 would, in turn, decrease the transcription of GLUT4 in insulin-sensitive tissues, such as skeletal muscle and adipose tissue, contributing to insulin resistance. On the other hand, although DIO2 is not known to be expressed in

### Table 1
Study population characteristics

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Age (years)</th>
<th>BMI (kg/m²)</th>
<th>Men/Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene scanning</td>
<td>50</td>
<td>38.5 ± 11</td>
<td>46.0 ± 5</td>
<td>11/39</td>
</tr>
<tr>
<td>Association analysis</td>
<td>972</td>
<td>56.0 ± 13</td>
<td>32.8 ± 8</td>
<td>71/901</td>
</tr>
<tr>
<td>(total)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Association analysis</td>
<td>135</td>
<td>42.3 ± 16.7</td>
<td>27.1 ± 7.8</td>
<td>0/135</td>
</tr>
</tbody>
</table>

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
</table>

Data are means ± SD and n.

### Table 2
Association of Thr92Ala DIO2 and glucose disposal euglycemic hyperinsulinemic clamp (n = 135 women)

<table>
<thead>
<tr>
<th></th>
<th>Thr/Thr</th>
<th>Thr/Ala</th>
<th>Ala/Ala</th>
<th>P (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>41.9 ± 2.3 (52)</td>
<td>43.9 ± 2.1 (66)</td>
<td>37.2 ± 4.0 (17)</td>
<td>0.33</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>72.3 ± 2.3 (52)</td>
<td>73.3 ± 2.1 (66)</td>
<td>74.3 ± 4.1 (17)</td>
<td>0.90</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.6 ± 0.8 (52)</td>
<td>27.4 ± 0.7 (66)</td>
<td>27.6 ± 1.4 (17)</td>
<td>0.70</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>4.6 ± 0.1 (51)</td>
<td>4.7 ± 0.1 (64)</td>
<td>4.8 ± 0.1 (17)</td>
<td>0.35</td>
</tr>
<tr>
<td>Fasting insulin (pmol/l)</td>
<td>57.0 ± 10.2 (49)</td>
<td>72.0 ± 9.6 (56)</td>
<td>105.0 ± 18.6 (16)</td>
<td>0.081</td>
</tr>
<tr>
<td>Glucose disposal (mmol/min)</td>
<td>22.6 ± 0.9 (52)</td>
<td>18.2 ± 0.8 (66)*</td>
<td>17.2 ± 1.6 (17)*</td>
<td>0.0006</td>
</tr>
<tr>
<td>Glucose disposal per kilogram fat free mass (mg · min⁻¹ · kg⁻¹)</td>
<td>0.54 ± 0.02 (52)</td>
<td>0.44 ± 0.02 (66)*</td>
<td>0.42 ± 0.04 (17)*</td>
<td>0.0088</td>
</tr>
</tbody>
</table>

*Data are means ± SEM (n). All variables adjusted for age. *P = 0.0001 for dominant model; †P = 0.0002 for dominant model. Thr/Thr = Thr92 homozygotes; Thr/Ala = heterozygotes; Ala/Ala = Ala92 homozygotes.
liver, we cannot rule out the possibility of a decreased hepatic insulin action.

Several studies have demonstrated that the Trp64Arg ADRB3 variant generates, upon stimulation, a reduced amount of cAMP compared to the wild-type receptor (22). Furthermore, we and others (23,24) have demonstrated that a functional cyclic AMP-responsive element is present in the promoter region of the DIO2 gene. The interaction between the Thr92Ala DIO2 and the Trp64Arg ADRB3 variants and a higher BMI could thus be due to a reduction in the transcription of an already functionally defective enzyme, ultimately generating a reduction of adrenergic-driven thyroid hormone-mediated lipolysis in adipose tissue. No significant difference was observed in thyroid function parameters between subjects with or without the Thr92Ala DIO2 variant in the subgroup of Italian obese subjects (data not shown).

This study has several strengths. First, the number of subjects studied was quite large, enabling us to examine gene/gene interactions. Furthermore, we had good power to detect relatively modest differences in phenotypes. Second, all subjects were Caucasian, thus reducing the risk of false positive/negative associations due to stratification bias. Third, a subset of individuals underwent euglycemic-hyperinsulinemic clamps, which enabled us to evaluate direct measures of insulin sensitivity rather than relying on indirect and less precise measures, as is the case in other studies of this kind. Despite these strengths, there were also limitations. First, the vast majority of the subjects in our study were women, and no men underwent euglycemic-hyperinsulinemic clamp. Thus, we do not know if these findings apply to men. Second, we cannot rule out the possibility of stratification bias and cannot comment on the generalizability of our findings to other ethnic populations. Third, without functional studies, it is possible that the Thr92Ala variant is in linkage disequilibrium with another pathogenic variant in the DIO2 gene or a gene close by. Finally, our results could represent a type 1 error. However, the P values for clamp measurements remains significant at the 0.01 level, even if Bonferroni corrected for all 29 variables in our database. This finding, along with the scientific plausibility of an association, provides evidence against type 1 error.

In conclusion, we have identified a novel common missense mutation of the DIO2 gene, which strongly associates with insulin resistance and, in the presence of Trp64Arg ADRB3, increased BMI. Further association and in vivo and in vitro studies are needed to evaluate the functional and epidemiological importance of this variant alone and combined with other candidate gene variants for obesity and insulin resistance.

### RESEARCH DESIGN AND METHODS

**Study population.** DIO2 molecular scanning was performed in 50 unrelated morbidly obese Caucasian subjects. Abnormal thyrotropin (>5 mUI/l) or secondary causes of obesity were considered as exclusion criteria. A well-characterized (20) Caucasian population of 972 unrelated subjects (91 men and 901 women participating in energy balance and molecular genetics studies of obesity) were used for association studies. Inclusion criteria were as follows: nondiabetic, nonsmokers, not taking any medication, without cardiovascular disease or hypertension. Glucose disposal rate, determined by hyperinsulinemic-euglycemic clamp (25) (insulin infusion rate 40 mU · m⁻² · min⁻¹) on a subset of 135 women, was performed as previously described (20). All study subjects provided informed consent.

**Mutation screening and genotype analysis.** The entire coding region, the intron-exon junctions, and the SECIS element of DIO2 were screened with

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Neither variant</th>
<th>Ala92 DIO2 only</th>
<th>Arg64 ADRB3 only</th>
<th>Both variants</th>
<th>P (interaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>364</td>
<td>474</td>
<td>52</td>
<td>70</td>
<td>1.000</td>
</tr>
<tr>
<td>Age (years)</td>
<td>55.7 ± 0.7</td>
<td>55.5 ± 0.6</td>
<td>58.3 ± 1.7</td>
<td>61.5 ± 1.5</td>
<td>0.501</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>87.5 ± 1.1</td>
<td>86.1 ± 1.0</td>
<td>83.2 ± 2.9</td>
<td>92.4 ± 2.5</td>
<td>0.011</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>33.1 ± 0.4</td>
<td>32.4 ± 0.4</td>
<td>31.8 ± 1.1</td>
<td>34.3 ± 0.9</td>
<td>0.041</td>
</tr>
</tbody>
</table>

*Data are means ± SE. Adjusted for age and sex. Subjects homozygous and heterozygous for each variant were reported together.

**FIG. 1.** Thr92Ala Dio2 polymorphism. A: PCR-SSCP. B: Dyodeoxy DNA sequencing. C: PCR-RFLP with Bsg-I. 1, Thr92 homozygote; 2, heterozygote; 3, Ala92 homozygote.
SSCP analysis. The gels (MDE, FMC, Rockland, ME) were run at four different conditions, i.e., 25°C or 4°C in the presence and absence of 10% glycerol. In our hands, the sensitivity of this method approaches 100%. The aberrantly migrating bands were confirmed with a second experiment. Direct dideoxy DNA sequencing on both strands of aberrantly migrating PCR products was performed manually according to established methods. The polymorphism was confirmed by PCR restriction fragment–length polymorphism (RFLP) analysis by digesting the PCR product resulting from a 5'TCTAGGCCCTG-GCAAAGTCAAG3' sense primer and a 5'CCACACTCTATTAGGCACATTTG3' antisense primer with BglI. The same PCR-RFLP strategy was used for population screening. PCR-RFLP of the Trp64Arg ADRB3 variant was performed as previously described (2).

Statistical analysis. Data were analyzed by age-adjusted and, where appropriate, sex-adjusted general linear models using SAS version 7.0 (SAS Institute, Cary, NC). To examine the main effect of the DIO2 Thr22Ala variant, the three genotypes (Thr/Thr, Thr/Ala, and Ala/Ala) were considered separately, followed by pooling the Thr/Ala and Ala/Ala groups when the glucose disposal data provided evidence of a dominant effect. In the analysis of interaction with Trp64Arg ADRB3, each of the two variants was modeled as dichotomous variables representing the presence or absence of at least one copy of the respective variant.

ACKNOWLEDGMENTS
The financial support of Telethon-Italy (Grant E.763) is gratefully acknowledged. F.S.C. is a recipient of the American Heart Association Grant 0160358U.

REFERENCES

D. MENTUCCIA AND ASSOCIATES

DIABETES, VOL. 51, MARCH 2002 883