Lack of Support for the Association between GAD2 Polymorphisms and Severe Human Obesity

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The demonstration of association between common genetic variants and chronic human diseases such as obesity could have profound implications for the prediction, prevention, and treatment of these conditions. Unequivocal proof of such an association, however, requires independent replication of initial positive findings. Recently, three (−243 A>G, +61450 C>A, and +83897 T>A) single nucleotide polymorphisms (SNPs) within glutamate decarboxylase 2 (GAD2) were found to be associated with class III obesity (body mass index > 40 kg/m²). The association was observed among 188 families (612 individuals) segregating the condition, and a case-control study of 575 cases and 646 lean controls. Functional data supporting a pathophysiological role for one of the SNPs (−243 A>G) were also presented. The gene GAD2 encodes the 65-kDa subunit of glutamic acid decarboxylase—GAD65. In the present study, we attempted to replicate this association in larger groups of individuals, and to extend the functional studies of the −243 A>G SNP. Among 2,359 individuals comprising 693 German nuclear families with severe, early-onset obesity, we found no evidence for a relationship between the three GAD2 SNPs and obesity, whether SNPs were studied individually or as haplotypes. In two independent case-control studies (a total of 680 class III obesity cases and 1,186 lean controls), there was no significant relationship between the −243 A>G SNP and obesity (OR = 0.99, 95% CI 0.83–1.18, p = 0.89) in the pooled sample. These negative findings were recapitulated in a meta-analysis, incorporating all published data for the association between the −243G allele and class III obesity, which yielded an OR of 1.11 (95% CI 0.90–1.36, p = 0.28) in a total sample of 1,252 class III obese cases and 1,800 lean controls. Moreover, analysis of common haplotypes encompassing the GAD2 locus revealed no association with severe obesity in families with the condition. We also obtained functional data for the −243 A>G SNP that does not support a pathophysiological role for this variant in obesity. Potential confounding variables in association studies involving common variants and complex diseases (low power to detect modest genetic effects, overinterpretation of marginal data, population stratification, and biological plausibility) are also discussed in the context of GAD2 and severe obesity.


Introduction

By dramatically increasing mortality [1] and morbidity [2] from cardiovascular disease, obesity has emerged as a major public health issue for the 21st century. Obesity is strongly associated with type 2 diabetes, hypertension, dyslipidemia, heart failure, and stroke [3]. This burden of disease is particularly high in individuals with class III obesity (body mass index [BMI] > 40 kg/m²), as they are more likely to develop at least one of these co-morbidities [4].

The importance of genetic factors in determining susceptibility to obesity has been well established elsewhere, by studies of twins [5], and adoptees [6]. At present, there is support for a model in which the propensity to become obese is determined largely by genetic factors, with environmental factors determining the expression of the condition [7]. These genetic influences are likely to be particularly powerful in individuals with severe or early-onset forms of obesity [8].

While several rare monogenic forms of non-syndromic obesity have been identified, the majority of individuals with severe obesity appear to be affected by common variations in complex loci. A potential candidate for such a role is the gene GAD2, which encodes the 65-kDa subunit of glutamic acid decarboxylase—GAD65. This enzyme plays a crucial role in the metabolism of GABA, a neurotransmitter that is involved in the control of food intake, energy expenditure, and body weight.

Among 2,359 individuals comprising 693 German nuclear families with severe, early-onset obesity, we found no evidence for a relationship between the three GAD2 SNPs and obesity, whether SNPs were studied individually or as haplotypes. In two independent case-control studies (a total of 680 class III obesity cases and 1,186 lean controls), there was no significant relationship between the −243 A>G SNP and obesity (OR = 0.99, 95% CI 0.83–1.18, p = 0.89) in the pooled sample. These negative findings were recapitulated in a meta-analysis, incorporating all published data for the association between the −243G allele and class III obesity, which yielded an OR of 1.11 (95% CI 0.90–1.36, p = 0.28) in a total sample of 1,252 class III obese cases and 1,800 lean controls. Moreover, analysis of common haplotypes encompassing the GAD2 locus revealed no association with severe obesity in families with the condition. We also obtained functional data for the −243 A>G SNP that does not support a pathophysiological role for this variant in obesity. Potential confounding variables in association studies involving common variants and complex diseases (low power to detect modest genetic effects, overinterpretation of marginal data, population stratification, and biological plausibility) are also discussed in the context of GAD2 and severe obesity.
obesity have been described to date [9–13], efforts aimed at identifying common susceptibility alleles for the condition have been much less successful [14].

The Chromosome 10p12 region has previously demonstrated significant linkage with severe human obesity [15]. In the initial study [15] involving individuals ascertained by a proband with class III obesity (BMI > 40 kg/m²) and at least one sibling with BMI > 27 kg/m², strong evidence for linkage (maximum logarithm of odds score 4.85) was obtained at the marker D10S197. The linkage peak encompassed a region of approximately 15 centimorgans. Confirmation of this linkage, albeit at lower levels of significance, was obtained in German Caucasians [16] and a combined sample of Caucasian Americans and African Americans [17]. The marker D10S197 is located within intron 7 of the glutamate decarboxylase 2 (GAD2) gene, which encodes the 65-kDa subunit of glutamic acid decarboxylase—GAD65.

Recently, Boutin et al. [18] obtained evidence to implicate GAD2 as a candidate gene for human obesity. In a case-control study for class III obesity, the authors identified both a haplotype (consisting of the most frequent alleles of single nucleotide polymorphisms [SNPs] +61450 C>A and +83897 T>A), and a SNP (−243 A>G) within GAD2 that differed in frequency between cases and controls. In family-based tests of association involving 612 individuals from 188 nuclear families, the +61450 C>A and +83897 T>A SNPs were associated with class III obesity. The “protective” wild-type (WT) haplotype (+61450 C and +83897 T) identified in the case-control study was found to be in excess in unaffected offspring.

As the GAD2 variant allele −243 G was in the 5’ region of the gene (the other two SNPs were located in intronic regions), displayed the strongest association with class III obesity in the case-control study, and was in linkage disequilibrium with the +61450 C>A and +83897 T>A SNPs, functional studies were performed to test its effects on transcription and nuclear protein binding. In a luciferase reporter gene containing the GAD2 promoter, the −243 G allele increased the transcriptional activity 6-fold relative to an equivalent reporter gene containing the WT (−243 A) allele in βTC3 murine insulinoma cells. Also, relative to the WT (A) allele, oligonucleotide probes containing the variant (G) allele had a higher affinity for an unidentified nuclear protein from βTC3 cells. Overall, their results suggested that the GAD2 −243 G allele might not only constitute a genetic marker for class III human obesity, but may also exert a significant physiological effect.

In recent years, many unreplicated associations have been reported between common genetic polymorphisms and measures of adiposity [19,20]. Indeed, one of the significant challenges in genetic association studies is the presence of statistical trends towards susceptibility (with the suspected allele being neither sufficient nor necessary for disease expression), rather than clear cause-and-effect relationships. This factor reduces the power of individual studies; consequently, it has become critical to develop larger multicenter studies to confirm positive associations in other populations, and to perform meta-analyses to more accurately estimate the magnitude of the genetic effect [21]. In the present study, we attempted to replicate the recent findings of Boutin et al. [18] by performing family-based tests of association and case-control studies in three Caucasian populations.

Results

Family-Based Tests of Association

In the previous report [18], an excess of WT alleles was observed in unaffected offspring for the +61450 C>A and +83897 T>A SNPs (p = 0.03 for each), and the haplotype consisting of the WT alleles at these SNPs was found to be in excess in unaffected offspring (p = 0.05). However, excess transmission of the G allele of the −243 A>G SNP to affected offspring was not observed (p = 0.06).

To further assess these initial findings in a much larger cohort, we performed family-based tests of association in 2,359 German Caucasian individuals from 693 nuclear families. Nuclear families were composed of obese children (mean BMI percentile = 98.6 ± 2.3, range 90th–100th percentile), their obese siblings, and both of their parents. The clinical characteristics of the nuclear families segregating obesity are shown in Table 1. This group of individuals included the 89 families that had previously displayed suggestive linkage for obesity (maximum likelihood binomial logarithm of odds score of 2.24) at D10S197 [22].

Using the pedigree disequilibrium test (PDT) [23], we found no evidence for excess transmission of any GAD2 alleles to obese children in the 89 families displaying prior linkage of obesity to Chromosome 10p (Table 2). This finding alone suggested that the original linkage signal in this region might be due to different SNPs than the ones under study. We next included samples that were not previously tested for linkage, bringing the total group to 693 nuclear families. As in the linked families, we found no association between any of the GAD2 SNPs and obesity (Table 2). Similarly, studies of haplotype transmission in the entire group using the transmission disequilibrium test (TDT) did not provide any evidence for a “protective” haplotype consisting of the +61450 C and +83897 T alleles, or any other two- or three-allele GAD2 haplotypes (Table 3).

Case-Control Studies

Previously [18], the −243 A>G, +61450 C>A, and +83897 T>A SNPs were associated with class III obesity in one case-control group (349 obese cases and 376 nonobese controls), whereas the association between the −243 A>G SNP and obesity was significant in the pooled sample of 575 obese cases and 646 controls (odds ratio [OR] of 1.3, 95% confidence interval [CI] 1.053–1.585, p = 0.014). We attempted to replicate these associations by performing case-control studies of class III obesity in two groups of North American Caucasians, one from the United States and the other from Canada. The clinical characteristics of the participants used in the case-control studies are shown in Table 1.

US case-control study. Each of the three GAD2 polymorphisms (−243 A>G, +61450 C>A, and +83897 T>A) was found to be in Hardy-Weinberg equilibrium in 302 class III obese (BMI > 40 kg/m²) cases and 427 lean controls (Table 4). None of the three variant alleles were associated with class III obesity (−243 G allele and class III obesity [OR = 1.11, 95% CI 0.84–1.46, p = 0.45]; the +61450 A allele [OR = 1.25, 95% CI 0.99–1.57, p = 0.058]; the +83897 A allele [OR = 1.14, 95% CI 0.87–1.50, p = 0.33]). Moreover, within the obese and lean groups, there was no association between GAD2 genotype and BMI for any of the three SNPs studied (unpublished data).

Canadian case-control study. The Canadian participants were also genotyped for the GAD2 −243 A>G, +61450 C>A,
and +83897 T>A SNPs. Genotypes of both cases and controls conformed to Hardy-Weinberg equilibrium, and the allele frequencies were similar to those observed in US Caucasians (Table 5).

As for US participants, the frequency of the −243 G allele did not differ between a group of 378 class III obese Canadian participants (frequency = 0.160) and a group of 759 lean controls (frequency = 0.175). The −243 G allele was not associated with class III obesity in this case-control study (OR = 0.90, 95% CI 0.71–1.14, p = 0.39). Pooling the results from the US and Canadian studies (680 class III obese cases and 1,186 lean controls) did not provide significant evidence for an association between the −243 G allele and class III obesity (OR = 0.99, 95% CI 0.83–1.18).

**Meta-analysis for the −243 A>G variant.** It has been proposed elsewhere [24] that the interpretation of results from association studies may be aided by meta-analysis of all similar studies. We compiled all available genotyping data (ours and that of the previous GAD2 study [18]) pertaining to the relationship between the −243 A>G polymorphism and class III obesity, and performed a meta-analysis (Figure 1). Inclusion of the data from the original study and our two case-control studies (a total of 1,252 cases and 1,800 controls) did not provide significant evidence for an association between the −243 G allele and class III obesity.

**Further Investigation of GAD2 as a Candidate Gene for Severe Obesity.**

In order to evaluate the potential relationship between other common SNPs in GAD2 and severe obesity, we conducted a comprehensive investigation of haplotype structure in this region using the data from the International HapMap Project (http://www.hapmap.org/cgi-perl/gbrowse/hapmap) and Haploview (http://www.broad.mit.edu/mpg/haplov/index.php) [25]. As described earlier by Boutin et al., GAD2 (and 2 kilobases of its promoter) lies within a 90-kilobase block of linkage disequilibrium on Chromosome 10p12 (Figure S1). Two of the three SNPs used in this study, −243 A>G (rs2236418) and +83897 T>A (rs928197), were used in the construction of HapMap. To incorporate the data from the third SNP, +61450 C>A (rs992990), into this framework, we genotyped the same CEPH (Centre D’Etude du Polymorphisme Humain) samples (Utah residents with ancestry from northern and western Europe) and the HapMap 1 (Utah residents with ancestry from northern and western Europe) that were used in the creation of the map. When the genotype results from the +61450 C>A SNP were integrated with those from HapMap, the overall structure of the haplotype block did not change. To capture at least 95% of the haplotype diversity within this haplotype block, we then determined that genotyping of three more SNPs was required: rs3781117 (intron 4), rs3781118 (intron 4), and rs1330581 (intron 7) (Figure S2).

The German families and case-control participants from the US and Canada were genotyped for the SNPs rs3781117, rs3781118, and rs1330581. None of these three SNPs were transmitted to affected children more frequently than expected by chance (Table S1). Inclusion of these GAD2 SNPs with the original three did not yield significant results for association between any GAD2 haplotype and obesity (Table 5).

### Table 1. Clinical Characteristics of Participants Used for Family-Based Association and Case-Control Studies

<table>
<thead>
<tr>
<th>Group</th>
<th>Sub-Group</th>
<th>n Participants</th>
<th>Sex (M/F)</th>
<th>Age (y) ± SD</th>
<th>BMI (kg/m²) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>German nuclear families</td>
<td>Parents</td>
<td>1,386</td>
<td>693/693</td>
<td>43 ± 6</td>
<td>30.4 ± 6.1</td>
</tr>
<tr>
<td></td>
<td>Children</td>
<td>973</td>
<td>433/540</td>
<td>14 ± 4</td>
<td>31.0 ± 6.0</td>
</tr>
<tr>
<td>US case-control</td>
<td>Class III obese</td>
<td>302</td>
<td>90/212</td>
<td>50 ± 12</td>
<td>48.5 ± 10.1</td>
</tr>
<tr>
<td></td>
<td>Lean controls</td>
<td>427</td>
<td>141/286</td>
<td>52 ± 5</td>
<td>22.9 ± 1.4</td>
</tr>
<tr>
<td>Canadian case-control</td>
<td>Class III obese</td>
<td>378</td>
<td>139/239</td>
<td>46 ± 10</td>
<td>47.6 ± 7.8</td>
</tr>
<tr>
<td></td>
<td>Lean controls</td>
<td>759</td>
<td>304/455</td>
<td>45 ± 15</td>
<td>20.2 ± 1.9</td>
</tr>
</tbody>
</table>

**DOI**: 10.1371/journal.pbio.0030315.t001

### Table 2. PDT Results for GAD2 SNPs in 693 German Families Segregating Severe, Early-Onset Obesity

<table>
<thead>
<tr>
<th>SNP</th>
<th>n Trios</th>
<th>Allele</th>
<th>Transmitted</th>
<th>Untransmitted</th>
<th>χ² (df)</th>
<th>p-Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>In the 89 Families Linked to Chromosome 10p</td>
<td>−243 A&gt;G</td>
<td>187</td>
<td>A</td>
<td>311</td>
<td>312</td>
<td>0.01 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>G</td>
<td>63</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+61450 C&gt;A</td>
<td>187</td>
<td>C</td>
<td>282</td>
<td>273</td>
<td>0.49 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>92</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+83897 T&gt;A</td>
<td>188</td>
<td>T</td>
<td>308</td>
<td>311</td>
<td>0.06 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>68</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>In All 693 Nuclear Families with Obesity</td>
<td>−243 A&gt;G</td>
<td>956</td>
<td>A</td>
<td>1,590</td>
<td>1,578</td>
<td>0.20 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>G</td>
<td>322</td>
<td>334</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+61450 C&gt;A</td>
<td>956</td>
<td>C</td>
<td>1,404</td>
<td>1,379</td>
<td>0.92 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>508</td>
<td>533</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+83897 T&gt;A</td>
<td>957</td>
<td>T</td>
<td>1,590</td>
<td>1,575</td>
<td>0.67 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>324</td>
<td>339</td>
<td></td>
</tr>
</tbody>
</table>

**DOI**: 10.1371/journal.pbio.0030315.t002
6). Moreover, rs3781117, rs3781118, and rs1330581 were not independently associated with class III obesity in either the US or Canadian case-control study groups (Table S2).

**Ethnic differences in GAD2 allele frequency.** The presence of an underlying population substructure, resulting from ethnic admixture, is a common bias in association studies [26]. We were therefore interested in determining whether different ethnic groups could display significant differences in GAD2 allele frequency.

In samples obtained from the Human Variation Collection (Coriell Institute for Medical Research, Camden, New Jersey, United States), the frequencies of GAD2 alleles in Caucasians were comparable with those observed for the Caucasian groups tested in previous studies (Table S3). However, we observed marked and highly significant differences in allele frequency for the −243 A>G SNP between Caucasians and populations of West African origin represented by samples collected in the US or in France. North African populations presented an intermediate allelic distribution.

**Reporter Gene Assay for GAD2 −243 G Promoter Variant**

In addition to the aforementioned genetic results, Boutin et al. [18] also found that luciferase reporter genes containing the −243 G allele in the GAD2 promoter (from −1710 to −4, relative to the transcriptional start site) displayed a 6-fold higher activity compared to reporter genes containing the −243A allele in βTC3 murine insulinoma cells (Figure S3 in [18]). We were interested in investigating the nature of this allele-specific difference in GAD2 promoter activity, with the goal of identifying the specific cis-acting elements responsible. To accomplish this, we also tested the effect of the −243 A>G SNP on transcription of a luciferase reporter gene in βTC3 cells.

We found that introduction of the −243 G allele into the −1710/−4 reporter gene did not elicit detectable effects on luciferase transcription relative to the WT reporter gene (Figure S3). Similarly, we could not detect any allele-specific effects of the −243 A>G polymorphism in two smaller reporter genes containing the GAD2 promoter (from −501 to −4 and from −1,234 to −4). However, the transcriptional activity of our WT −1710/−4 reporter gene was appreciably higher than that of pGL3Basic in βTC3 cells, suggesting that the GAD2 promoter does exhibit some basal transcriptional activity in this cell line.

**Table 3. Analysis of Haplotype Transmission in German Obesity Trios Using the TDT**

<table>
<thead>
<tr>
<th>−243 A&gt;G</th>
<th>+61450 C&gt;A</th>
<th>+83897 T&gt;A</th>
<th>Transmitted/Untransmitted (% Transmitted)</th>
<th>( \chi^2 )</th>
<th>p-Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>C</td>
<td>-</td>
<td>225/213 (51.4%)</td>
<td>0.33</td>
<td>0.57</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>-</td>
<td>112/113 (49.8%)</td>
<td>0.00</td>
<td>0.95</td>
</tr>
<tr>
<td>G</td>
<td>C</td>
<td>-</td>
<td>16/14 (53.3%)</td>
<td>0.13</td>
<td>0.72</td>
</tr>
<tr>
<td>G</td>
<td>A</td>
<td>-</td>
<td>135/148 (47.7%)</td>
<td>0.60</td>
<td>0.44</td>
</tr>
<tr>
<td>-</td>
<td>C</td>
<td>T</td>
<td>228/212 (51.8%)</td>
<td>0.58</td>
<td>0.45</td>
</tr>
<tr>
<td>-</td>
<td>C</td>
<td>A</td>
<td>0/3 (0%)</td>
<td>3.00</td>
<td>0.08</td>
</tr>
<tr>
<td>-</td>
<td>A</td>
<td>T</td>
<td>106/105 (50.2%)</td>
<td>0.00</td>
<td>0.95</td>
</tr>
<tr>
<td>-</td>
<td>A</td>
<td>A</td>
<td>156/170 (47.9%)</td>
<td>0.60</td>
<td>0.44</td>
</tr>
<tr>
<td>A</td>
<td>C</td>
<td>T</td>
<td>227/212 (51.7%)</td>
<td>0.51</td>
<td>0.47</td>
</tr>
<tr>
<td>A</td>
<td>C</td>
<td>A</td>
<td>0/2 (0%)</td>
<td>2.00</td>
<td>0.16</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>T</td>
<td>100/94 (51.5%)</td>
<td>0.19</td>
<td>0.67</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>A</td>
<td>19/25 (43.2%)</td>
<td>0.82</td>
<td>0.37</td>
</tr>
<tr>
<td>G</td>
<td>C</td>
<td>T</td>
<td>15/13 (53.6%)</td>
<td>0.14</td>
<td>0.71</td>
</tr>
<tr>
<td>G</td>
<td>C</td>
<td>A</td>
<td>0/1 (0%)</td>
<td>1.00</td>
<td>0.32</td>
</tr>
<tr>
<td>G</td>
<td>A</td>
<td>T</td>
<td>5/6 (45.5%)</td>
<td>0.09</td>
<td>0.76</td>
</tr>
<tr>
<td>G</td>
<td>A</td>
<td>A</td>
<td>133/146 (47.7%)</td>
<td>0.61</td>
<td>0.44</td>
</tr>
</tbody>
</table>

All possible two- and three-allele haplotypes for the GAD2 SNPs −243A>G, +61450C>A, and +83897T>A are shown. Results for the “protective” haplotype, consisting of the +61450C and +83897T alleles, are shown in bold. DOI: 10.1371/journal.pbio.0030315.t003

**Table 4. Genotype Results for US Case-Control Study**

<table>
<thead>
<tr>
<th>SNP</th>
<th>n (Frequency)</th>
<th>Allele Frequency</th>
<th>( \chi^2(\text{df}) )</th>
<th>p-Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>−243 A&gt;G</td>
<td>Cases</td>
<td>203 (0.67)</td>
<td>87 (0.29)</td>
<td>12 (0.04)</td>
</tr>
<tr>
<td>Controls</td>
<td>293 (0.69)</td>
<td>124 (0.29)</td>
<td>10 (0.02)</td>
<td>0.831</td>
</tr>
<tr>
<td>+61450 C&gt;A</td>
<td>Cases</td>
<td>148 (0.49)</td>
<td>120 (0.40)</td>
<td>34 (0.11)</td>
</tr>
<tr>
<td>Controls</td>
<td>228 (0.53)</td>
<td>171 (0.40)</td>
<td>28 (0.07)</td>
<td>0.734</td>
</tr>
<tr>
<td>+83897 T&gt;A</td>
<td>Cases</td>
<td>198 (0.66)</td>
<td>92 (0.30)</td>
<td>12 (0.04)</td>
</tr>
<tr>
<td>Controls</td>
<td>291 (0.68)</td>
<td>125 (0.29)</td>
<td>11 (0.03)</td>
<td>0.828</td>
</tr>
</tbody>
</table>

The GAD2 SNPs −243A>G, +61450C>A, and +83897T>A are also referred to as rs2236418, rs992990, and rs928197 in the text. DOI: 10.1371/journal.pbio.0030315.t004
Electrophoretic Mobility Shift Assay

In the previous study [18], oligonucleotide probes containing either of the −243 A>G alleles were tested for their affinity for nuclear extract prepared from βTC3 murine insulinoma cells. The probe containing the −243 G allele was found to have a 6-fold higher affinity for an unidentified nuclear protein (Figure 4 in [18]). However, the DNA–protein complex was also present to some extent in the negative control lanes (lacking nuclear extract), and the oligonucleotide probes differed with respect to their specific activity. We obtained the sequences of oligonucleotide probes used by the authors, and utilized the electrophoretic mobility shift assay (EMSA) to confirm this allele-specific difference in binding affinity. We were also interested in determining whether this effect was specific for neuronal or β cells relative to other cell lines. Our experiments indicated that the −243 A allele had a greater affinity for an unidentified protein from βTC3 nuclear extracts relative to the −243 G allele (Figures S4 and S5). Our results were not consistent with those previously described by Boutin et al. [18].

Table 5. Genotype Results for Canadian Case-Control Study

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype, n (Frequency)</th>
<th>Allele Frequency</th>
<th>( \chi^2(\text{df}) )</th>
<th>( p )-Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>−243 A&gt;G</td>
<td>Cases: 266 (0.70) 103 (0.27) 9 (0.03)</td>
<td>A: 0.840  G: 0.160</td>
<td>0.76 (1)</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>Controls: 523 (0.69) 207 (0.27) 29 (0.04)</td>
<td>A: 0.825  G: 0.175</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+61450 C&gt;A</td>
<td>Cases: 199 (0.53) 144 (0.38) 32 (0.09)</td>
<td>A: 0.723  C: 0.277</td>
<td>0.97 (1)</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>Controls: 381 (0.50) 299 (0.40) 75 (0.10)</td>
<td>A: 0.703  C: 0.297</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+83897 T&gt;A</td>
<td>Cases: 248 (0.67) 106 (0.29) 15 (0.04)</td>
<td>A: 0.816  T: 0.184</td>
<td>0.11 (1)</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>Controls: 484 (0.66) 224 (0.30) 28 (0.04)</td>
<td>A: 0.810  T: 0.190</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The GAD2 SNPs −243A>G, +61450C>A, and +83897T>A are also referred to as rs2236418, rs992990, and rs928197 in the text.
DOI: 10.1371/journal.pbio.0030315.i005

Figure 1. Meta-Analysis for the Association between the GAD2 −243 A>G Polymorphism and Class III Obesity

French groups 1 and 2 refer to the genotype results from two sets of Caucasian class III obese cases and controls studied by Boutin et al. [18]. US and Canadian groups were from the present study. The meta-analysis for the association between the −243 G allele and class III obesity yielded a summary OR of 1.11 (95% CI 0.90–1.36), obtained in a total sample of 1,252 class III obese cases and 1,800 controls using a Mantel-Haenszel method and a fixed effects model.
DOI: 10.1371/journal.pbio.0030315.g001
**Discussion**

In the present study, we attempted to replicate the important recent findings of Boutin et al. [18], which implicated three SNPs in GAD2 (the −243 A>G allele and a haplotype of the +61450 C>A and +83897 T>A SNPs) in the predisposition to class III human obesity. To replicate their findings, we first performed family-based tests of association for all three SNPs in 693 nuclear families segregating severe obesity (2,359 participants, nearly four times as many participants as in the original report). This group of individuals included 89 families found to have linkage of severe obesity to Chromosome 10p12 [16,22]. No evidence for excess transmission of any GAD2 alleles or haplotypes from parents to affected offspring was obtained. Next, we conducted an adequately powered case-control study to test the association between class III obesity and the GAD2 −243 A>G variant in Caucasians. Consistent with the family-based association results, we did not observe any association between the −243 G variant and class III obesity (680 cases and 1,186 lean controls). These findings were also obtained in a meta-analysis for the association between the −243 A>G SNP and class III obesity. Lastly, we obtained results from the reporter gene and DNA binding experiments for the −243 A>G variant that were inconsistent with the original report. Overall, we found that (i) a haplotype consisting of the WT alleles at SNPs +61450 C>A and +83897 T>A does not appear to protect against severe, early-onset obesity, (ii) the −243 A>G SNP is not associated with class III obesity in adults, (iii) other haplotypes in the region of GAD2 are not associated with severe obesity, and (iv) the −243 A>G SNP does not elicit detectable effects on transcription of a luciferase reporter gene in βTC3 murine insulinoma cells.

Irreproducibility of positive findings has been a common criticism leveled at association studies investigating the common genetic basis of complex diseases [19,24]. The reasons cited are numerous, and include a lack of statistical power to detect small to moderate effects, lack of control over the Type I error rate, overinterpretation of marginal data, population stratification, and poor biological plausibility [27,28]. Regarding the conflicting results obtained by Boutin et al. [18] and the current study, it is likely that the lack of replication could be ascribed to any of these causes, which are discussed below. The inconsistencies between association studies may also reflect the complex interactions between multiple population-specific genetic and environmental factors.

The lack of statistical power to detect alleles of minor effect is likely to have contributed to the differences between the study by Boutin et al. [18] and the current investigation. Based on the findings of the initial report, we conducted an adequately powered, ethnically matched, case-control study. Although our results overlapped with the size of the initial effect, they did not show a significant association between the −243 G allele and class III obesity (Figure 1). We estimate that we had 60% power to detect a significant difference (α of 0.05) in allele frequency between our pooled groups of cases and controls, assuming that the −243 G allele (frequency of 0.18) was the disease allele, a genotype relative risk of 1.25, and a prevalence of class III obesity in the general population of 5% [29]. The family-based association tests had a similar amount of power (~60%), given the same assumptions.

Under these conditions, the original study [18] may have been underpowered. Moreover, it must be pointed out that the marginally significant association (p = 0.04) they observed between the −243 G allele and class III obesity was observed in only one of their two groups of participants, and did not reach nominal significance in their family-based analysis (p = 0.06). Although the lack of statistical significance does not exclude the possibility of an association (as we cannot rule out smaller effects), the data do not support a relationship between this SNP and class III obesity.

The interpretation of results from genetic association studies is frequently complicated by other statistical issues, such as a failure to control for multiple hypothesis testing, overinterpretation of marginal data as positive trends, and the well-documented tendency for initial positive findings to overestimate the strength of the association [21]. This “jackpot” phenomenon [24] can be readily observed in our meta-analysis (Figure 1).

Population stratification may also account for some of the inconsistencies observed between association studies, though its importance may have been overestimated [19,26]. Population stratification is usually controlled for by careful matching of cases and controls by ethnicity, using family-based tests of association (such as the TDT) or studying multiple case-control populations [30]. Considering the marked differences in allele frequency that we observed between ethnic groups for the GAD2 SNPs (the −243 A>G and +61450 C>A SNPs in particular), as well as the known differences in the prevalence of class III obesity between Caucasian Americans and African Americans [31], it is plausible that a small difference in ancestry between cases

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**Table 6. TDT Results for Six SNPs Spanning the GAD2 Haplotype Block**

<table>
<thead>
<tr>
<th>rs2236418</th>
<th>rs3781118</th>
<th>rs3781117</th>
<th>rs1330581</th>
<th>rs992990</th>
<th>rs928197</th>
<th>Transmitted/</th>
<th>Untransmitted</th>
<th>χ² (df)</th>
<th>p-Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>G</td>
<td>T</td>
<td>G</td>
<td>A</td>
<td>T</td>
<td>105/101(51.0%)</td>
<td>0.08 (1)</td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>T</td>
<td>C</td>
<td>A</td>
<td>C</td>
<td>T</td>
<td>142/144(49.7%)</td>
<td>0.01 (1)</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>T</td>
<td>T</td>
<td>A</td>
<td>C</td>
<td>T</td>
<td>722/705(50.6%)</td>
<td>0.20 (1)</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>T</td>
<td>T</td>
<td>G</td>
<td>A</td>
<td>A</td>
<td>184/190(49.2%)</td>
<td>0.10 (1)</td>
<td>0.76</td>
<td></td>
</tr>
</tbody>
</table>

Global likelihood ratio test

|        |        |        |        |        |        |        | 1.56 (4) | 0.82 |

Results were obtained using the program UNPHASED [49]. Rare haplotypes (frequency < 0.05) were excluded from the analysis. The SNPs rs2236418, rs992990, and rs928197 refer to the GAD2 SNPs −243 A>G, +61450 C>A, and +83897 T>A, respectively.

DOI: 10.1371/journal.pbio.0030315.t006
and controls could lead to spurious claims of association. Naturally, future studies of the GAD2 gene should carefully take this into consideration.

There is no obvious explanation for the differences in results obtained for the EMSA and reporter gene assays. Regarding the EMSA, a major problem with these experiments is that most random DNA sequences will be bound by a nuclear extract from any cell line (Figures S4 and S5 and Figure 4 in [18]). It is likely that the introduction of single base-pair differences into this DNA sequence will interfere with the binding pattern observed. Moreover, while an allelic-specific difference in the binding of βT3C cell nuclear extract definitely occurs for the −243 A>G polymorphism, this observation is of limited physiological significance, because: (i) it appears to be restricted to this cell type (and there is no apparent difference in allele-specific binding for nuclear extract derived from a neuronal cell line); and (ii) the binding of this nuclear protein does not appear to affect transcription of a luciferase reporter gene in βT3C cells. Finally, even if the −243 A>G SNP did affect transcription of the reporter gene in this context, there is no prior biological evidence to suggest that perturbation of GAD2 expression in β cells could exert detectable effects on long-term energy homeostasis.

This latest point raises the issue of biological plausibility. GAD2 encodes the 65-kDa isofrom of the enzyme glutamate decarboxylase, which catalyzes the production of γ-amino- butyric acid (GABA), a major inhibitory neurotransmitter, from glutamic acid. The biological evidence implicating GAD2 as a candidate gene (and by extension, hypothalamic GABA levels as causative) in severe obesity is as follows: GAD2 mRNA is co-expressed with neuropeptide Y in neurons of the hypothalamic arcuate nucleus that act in the nearby paraventricular nucleus and other hypothalamic areas to stimulate food intake [32]. Concomitantly, these arcuate neuropeptide Y neurons inhibit the parallel and opposing effects of neighboring pro-opiomelanocortin/cocaine- and amphetamine-regulated transcript neurons via GABA-ergic effects of neighboring pro-opiomelanocortin/cocaine- and amphetamine-regulated transcript neurons via GABA-ergic

GAD2 Polymorphisms and Obesity

Participants. The ascertainment strategy for these participants has been described previously [43]. BMI was calculated as weight in kg/(height in m)². For the PPT and TDT analyses, we genotyped 973 (extremely) obese children and adolescents (693 probands and 280 of their siblings; mean age 14.0 ± 3.7 y, mean BMI 31.0 ± 6.0 kg/m²) and both of their parents (mean age 42.7 ± 5.9 y, mean BMI 30.4 ± 6.1 kg/m²). Written informed consent was given by all participants and, in the case of minors, their parents. The Ethics Committee of the University of Marburg approved the study.

Participants were selected from the Cardiovascular Research Institute Genomic Resource in Arteriosclerosis, a population-based investigation of dyslipidemia and atherosclerotic heart disease established at the University of California, San Francisco (UCSF) in California, United States. This population includes patients from the Lipid Clinic of UCSF [44,45], from the UCSF Interventional Cardiology Service, and from collaborating cardiology clinics throughout California. The UCSF Committee on Human Research approved the protocols, and informed written consent was obtained from all patients. From this study group, we selected class III obese and 241 bp; G-allele: undigested). To detect rs928197 (trans-prenyltransferase), the enzyme that elongates the prenyl side-chain of coenzyme Q, one of the key elements of the respiratory chain within mitochondria; GPR158, which encodes a metabotropic glutamate, GABA₆-like G-protein–coupled receptor; and PTF1A, which encodes pancreas-specific transcription factor 1a. Although only a little is known about each of these genes, it is possible to speculate on the potential role of each in obesity. GAD2 is no exception. At present, however, there is insufficient genetic or biological evidence to implicate genetic variation in GAD2 in the predisposition to severe obesity in humans.

Materials and Methods

Genotyping.

Three GAD2 SNPs were genotyped by PCR-based restriction fragment length polymorphism analysis or by tetra-amplification refractory mutation system PCR. To detect rs61418 (−243 A>G), a PCR-amplicon of 636 base pairs (bp) (primers: GAD2–243-F: 5’-GGAGGCCCAGCTCAACAAA-3’ and GAD2–243-R: 5’-TTTGGAAGACTGGAGCGGTC-3’) was digested by DraI [New England Biolabs (NEB)]. Obese Caucasian individuals with a mean BMI of 48 kg/m² (range 36–81 kg/m², with 91% of individuals having a BMI > 40 kg/m²) were recruited from the Ottawa Hospital Weight Management Clinic. Age- and sex-matched lean Caucasian individuals with a BMI below the 10th percentile for age and sex were recruited as controls from the Ottawa region. The Human Ethics Research Boards of the Ottawa Hospital and the University of Ottawa Heart Institute approved the study. Informed written consent was obtained from all participants.

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199-bp amplicon detects the C-allele; GAD2-1710/4 construct. PCR was performed using TaKaRa LA Taq according to the manufacturer’s instructions (TaKaRa Biomedicals, Otsu, Shiga, Japan). Using restriction enzyme digestion of the WT and mutant constructs, the GAD2-1710/4 construct was digested into two fragments. The fragment sizes were determined using agarose gel electrophoresis.

After communication with the author, P. Boutin, we amplified the 199-bp amplicon using the primers GAD2PROM3 (our designation) CCGGGTACCCGGGTCTCCTGTTTGTAGC and GAD2PROM3 CAAGCTTGGAGACCTGGAGCAAG, digested the PCR product with KpnI and HindIII, and inserted it into the KpnI and HindIII sites in front of the firefly luciferase coding sequence, contained in the vector pGLO3 (Promega, Madison, Wisconsin, United States). This vector was referred to hereafter as the GAD2-1710/4 construct (numbers refer to the regions of the GAD2 promoter, relative to the transcriptional start site). The –243 G variant allele was introduced into this construct by PCR amplification of the above fragment from a heterozygous patient, digesting of this PCR product with NcoI and HindIII, and substitution of this fragment into the NcoI/HindIII sites of the WT construct. PCR was performed using TaKaRa LA Taq according to the manufacturer’s instructions (TaKaRa Biomedicals, Otsu, Shiga, Japan). Using restriction enzyme digestion of the WT and mutant constructs, the GAD2-1710/4 construct was digested into two fragments. The fragment sizes were determined using agarose gel electrophoresis. Five hundred nanograms of each forward (F) oligonucleotide were end-labeled with γ-32P ATP (Perkin-Elmer, Boston, Massachusetts, United States) using T4 polynucleotide kinase (Promega) at 37°C for 30 min. Subsequently, 1.5 μg of the corresponding unlabelled reverse (R) oligonucleotide and 50 μl of annealing buffer (100 mM NaCl in TE buffer) were added to each labeled (F) oligonucleotide, and the mixture was incubated for 10 min at 95°C before being cooled slowly for 1–2 h. The resulting labeled, double-stranded probe was then column-purified (Stratagene NucTrap, La Jolla, California, United States), and the concentration of the probe in the eluate was assessed using a 10 ng/μl standard. The probes for competition experiments were also prepared in a similar manner.

All EMSA experiments were performed in a 20-μl reaction volume containing binding buffer (10 mM HEPES [pH 7.9], 75 mM KCl, 2.5 mM MgCl2, 0.1 mM EDTA, 50 μl of annealing buffer (100 mM NaCl in TE buffer)) and 5 μg of labeled probe. Nuclear extracts from BT3C, NeuroA2, T98G, HepG2, and HEK293 cells were prepared using the method of Shen et al. [51]. Cells from which nuclear extracts were prepared were maintained in a water-jacketed incubator set to 37°C with 5% carbon dioxide. The murine insulinoma cell line (INS-1) were prepared using the method of Liao et al. [52]. All other materials were prepared using the method of Schreiber et al. [51]. Cells from which nuclear extracts were prepared were maintained as described below. After addition of the probe, the mixture was incubated for 10 min at room temperature before loading onto a 5% nondenaturing acrylamide gel containing 0.5 X TBE (1 X Tris-Borate EDTA) for 2 h. After blotting, membranes were probed with a 32P-labeled oligonucleotide containing the GAD2 proximal promoter sequence.

Supporting Information

Figure S1. Haplowiew of the GAD2 Region on Chromosome 10

This figure was generated using data from the International HapMap Project (http://www.hapmap.org/cgi-perl?gbrowse?gbrowse/hapmap) and using the program Haploview (http://www.broad.mit.edu/mpg/haplovie/index.php). The SNPs studied are indicated on the map by the following numbers: rs2291418 (#2); rs3787118 (#3); rs3787117 (#4), rs1330581 (#12), and rs928107 (#34). On the diagram, the blue squares indicate missing data and unfilled red squares indicate a high degree of linkage disequilibrium (linkage disequilibrium coefficient, D’).
D' = 1) between pairs of markers. Lesser degrees of linkage disequilibrium are indicated by the lighter red shading.

Found at DOI: 10.1371/journal.pbio.0030315.sg001 (95 KB PPT).

**Figure S2.** Haplotype Tag SNPs Required to Capture > 95% of the Haplotype Diversity within the GAD2 Region

The SNPs genotyped in the initial phase of the study (−243 A>G/ rs2294118, −601 G>A/ rs1889990, and +88397 T>A/ rs281957) are indicated on the upper part of the diagram as markers 2, 24, and 35, respectively. Haplotypes are depicted as rows, with their population frequency shown at the right side of each row. The SNPs that are in complete linkage disequilibrium with each other are shaded the same color. In order to determine > 95% of the haplotype information within the GAD2 region, genotypes at each of the SNPs indicated by the arrowheads (markers 1, 3, 4, 12, and 14, or a marker in perfect linkage disequilibrium with each) were required. To accomplish this, markers rs7811118 (*3 on diagram), rs7811117 (*4), and rs1330581 (*12) were genotyped in the second phase of the study.

Found at DOI: 10.1371/journal.pbio.0030315.sg002 (81 KB PPT).

**Figure S3.** Results from Transient Transfection of GAD2 Reporter Genes in TC3 Cells Containing the −243 A>G Polymorphism

Three different sizes of luciferase reporter gene were constructed from the GAD2 promoter (−1710→−501→−1294→−4) for transfection into TC3 murine insulinoma cells. Each WT reporter construct contains the −243 A allele, and the corresponding mutant reporter construct is identical to the WT except for the introduction of the −245 G allele. Twenty-four h before transfection, TC3 cells were seeded in 6-well plates at a density of 250,000 cells/well containing DMEM supplemented with 10% fetal calf serum (Hyclone, Logan, Utah, United States), 2 mM L-glutamine, and penicillin/streptomycin. On the day of the experiment, each well was transfected with pGL3Basic or a GAD2 promoter construct (0.4 μg) as well as 20 μg of the plasmid pRL-RSV (Promega), which encodes Renilla luciferase, to control for transfection efficiency. Transfections were performed in triplicate using Effectene reagent (Qiagen, Valencia, California, United States). Forty-eight h after transfection, cells were lysed, and firefly and Renilla luciferase assays were performed on the lysate using the Dual Luciferase Reporter Assay System (Promega), according to the manufacturer’s standard protocol. Each experiment was repeated three times. We observed no significant difference in luciferase activity between each pair of WT and mutant GAD2 promoter constructs.

Found at DOI: 10.1371/journal.pbio.0030315.sg003 (36 KB PPT).

**Figure S4.** EMSA

Radiolabeled double-stranded oligonucleotide probes for each of the −243 A>G alleles were incubated with various nuclear extracts and electrophoresed in a 5% nondenaturing polyacrylamide gel. The complexes formed by the interaction between the radiolabeled double-stranded oligonucleotide probes for each of the GAD2 region, genotypes at each of the SNPs indicated by the arrowheads (markers 1, 3, 4, 12, and 14, or a marker in perfect linkage disequilibrium with each) were required. To accomplish this, markers rs7811118 (*3 on diagram), rs7811117 (*4), and rs1330581 (*12) were genotyped in the second phase of the study.

Found at DOI: 10.1371/journal.pbio.0030315.sg004 (2.5 MB PPT).

**Figure S5.** PDT Results for rs3781117, rs3781118, and rs1330581

Comparison of luciferase activity between each pair of WT and mutant GAD2 promoter constructs.

Found at DOI: 10.1371/journal.pbio.0030315.sg005 (101 KB PPT).

**Table S1.** PDT Results for rs3781117, rs3781118, and rs1330581

None of the variant alleles at any of these three SNPs were associated with class III obesity in either of the two case-control groups or when pooled (for rs781117, C allele: OR = 0.92, 95% CI 0.75–1.13, p = 0.43; for rs781118, G allele: OR = 0.94, 95% CI 0.75–1.19, p = 0.62; for rs1330581, G allele: OR = 1.03, 95% CI 0.89–1.20, p = 0.65).

Can, Cases, Canadian cases.

Found at DOI: 10.1371/journal.pbio.0030315.stat002 (64 KB DOC).

**Table S3.** Genotype results for GAD2 SNPs in US Caucasians, African Americans, and Africans

Differences in allele frequency between ethnic groups were assessed by χ² test. For the −243 A>G and −41450 C>A SNPs, the differences between Caucasian Americans (CA) and African Americans were significantly different (CA vs. WA, p = 0.001; CA vs. NA, p = 0.014; WA vs. NA, p < 0.001). The frequency of the +88397 T>A SNP was also significantly different between CA and WA (p = 0.013). Other comparisons either yielded non-significant results, or were not conducted, as samples did not conform to Hardy-Weinberg equilibrium (indicated by an asterisk).

Found at DOI: 10.1371/journal.pbio.0030315.stat003 (53 KB DOC).

**Accession number**


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**Competing interests.** The authors have declared that no competing interests exist.

**Author contributions.** MMS, BW, LAP, DLL, AH, JH, and CV conceived and designed the experiments. MMS, BW, DLL, and AU performed the experiments. MMS, BW, DLL, MMC, FG, AS, and WCH analyzed the data. LAP, MMC, RM, MM, WR, FMJ, CRP, JPK, RD, RM, PYK, AH, and JH contributed reagents/materials/analysis tools. MMS, LAP, AH, JH, and CV wrote the paper.

**References**