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a randomised trial of hypo-energetic high- versus low-fat diets

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Allelic Variants of Melanocortin 3 Receptor Gene (MC3R) and Weight Loss in Obesity: A Randomised Trial of Hypo-Energetic High- versus Low-Fat Diets

José L. Santos1,2, Rolando De la Cruz3, Claus Holst4, Katrine Grau4, Carolina Naranjo3, Alberto Maiz1, Arne Astrup5, Wim H. M. Saris6, Ian MacDonald8, Jean-Michel Oppert7, Torben Hansen9, Oluf Pedersen9,10, Thorkild I. A. Sorensen4, J. Alfredo Martinez2*, NUGENOB Consortium

1 Department of Nutrition, Diabetes and Metabolism, Pontificia Universidad Católica de Chile, Santiago, Chile, 2 Department of Nutrition and Food Sciences, Physiology and Toxicology, University of Navarra, Pamplona, Spain, 3 Department of Public Health and Department of Statistics, Pontificia Universidad Católica de Chile, Santiago, Chile, 4 Centre for Health and Society, Institute of Preventive Medicine, Copenhagen University Hospital, Copenhagen, Denmark, 5 Department of Human Nutrition, Faculty of Life Science, University of Copenhagen, Copenhagen, Denmark, 6 Department of Human Biology, Nutrition and Toxicology Research Institute Maastricht, Maastricht University, Maastricht, The Netherlands, 7 Department of Nutrition, Hotel-Dieu Hospital University Pierre-et-Marie Curie (Paris 6), Human Nutrition Research Center Ile-de-France, Paris, France, 8 School of Biomedical Science, University of Nottingham Medical School, Queen’s Medical Centre, Nottingham, United Kingdom, 9 Hagedorn Research Institute, Gentofte, Denmark, 10 Institute of Biomedical Science, University of Copenhagen, Copenhagen, Denmark

Abstract

Introduction: The melanocortin system plays an important role in energy homeostasis. Mice genetically deficient in the melanocortin-3 receptor gene have a normal body weight with increased body fat, mild hypophagia compared to wild-type mice. In humans, Thr6Lys and Val81Ile variants of the melanocortin-3 receptor gene (MC3R) have been associated with childhood obesity, higher BMI Z-score and elevated body fat percentage compared to non-carriers. The aim of this study is to assess the association in adults between allelic variants of MC3R with weight loss induced by energy-restricted diets.

Subjects and Methods: This research is based on the NUGENOB study, a trial conducted to assess weight loss during a 10-week dietary intervention involving two different hypo-energetic (high-fat and low-fat) diets. A total of 760 obese patients were genotyped for 10 single nucleotide polymorphisms covering the single exon of MC3R gene and its flanking regions, including the missense variants Thr6Lys and Val81Ile. Linear mixed models and haplotype-based analysis were carried out to assess the potential association between genetic polymorphisms and differential weight loss, fat mass loss, waist change and resting energy expenditure changes.

Results: No differences in drop-out rate were found by MC3R genotypes. The rs6014646 polymorphism was significantly associated with weight loss using co-dominant (p = 0.04) and dominant models (p = 0.03). These p-values were not statistically significant after strict control for multiple testing. Haplotype-based multivariate analysis using permutations showed that rs3827103–rs1543873 (p = 0.06), rs6014646–rs6024730 (p = 0.05) and rs3746619–rs3827103 (p = 0.10) displayed near-statistical significant results in relation to weight loss. No other significant associations or gene*diet interactions were detected for weight loss, fat mass loss, waist change and resting energy expenditure changes.

Conclusion: The study provided overall sufficient evidence to support that there is no major effect of genetic variants of MC3R and differential weight loss after a 10-week dietary intervention with hypo-energetic diets in obese Europeans.

Introduction

Obesity and hyperphagia displayed by animal models with genetic alterations in the leptin-melanocortin system outline the importance of these biological pathways in body weight regulation [1]. Genetic ablation of melanocortin 4 receptor gene in mice produces hyperphagia and obesity [2] while rare mutations in the corresponding human MC4R are a cause of severe childhood obesity [3]. Contrary to the effects observed in mice Mc4r gene, the Mc3r+/− mouse shows an unique phenotype characterized by higher percentage of body fat without increased body weight, mild hypophagia, higher energy efficiency, reduced locomotor activity, hyperleptinemia and reduced linear growth compared with the Mc3r++/+ mouse [4,5]. Indeed, it has been reported that mice with genetic disruption in both Mc3r and Mc4r are heavier than Mc4r−/− mice, indicating a possible non-redundant
participation of both melanocortin receptors in obesity and adiposity-related phenotypes [4].

Some linkage studies are concordant with the presence of a susceptibility gene for human obesity at the melanocortin-3 receptor MC3R locus (gene ID:4159; 20q13.2-13.3) [6,7]. The missense variants Thr6Lys (rs3746619) and Val81Ile (rs3827105) of MC3R have been related with in vitro diminished functionality and expression of the receptor, showing a significant association with childhood obesity in a case-control study [8]. Additionally, some mutations in MC3R have been proposed as a cause of human monogenic obesity [9]. Genetic epidemiological studies have reported different results regarding the association between common MC3R variants and common forms of obesity [10–14]. Additionally, large genome-wide association studies have not been able to substantiate associations between variants near the MC3R locus and polygenic obesity [15]. In this context, there is published evidence to support a role of the MC3R variants Thr6Lys and Val81Ile in weight loss responses of obese children [16]. Herein, we have used previously published data from the NUGENOB study (http://www.nugenob.org) (Figure 1) [17] to assess the association between genetic variants of MC3R with weight loss induced by hypo-energetic low-fat/high-fat diets in obese European adults.

Methods

Ethics Statement

The selection of participants was carried out in the frame of the NUGENOB project (http://www.nugenob.org) [17], under the approval of Ethical committees at each of the participating centers. Volunteers were informed about the nature of the study, and written consent was obtained prior to study participation. The names of the ethics local committees were: Research Ethic Committee of the University of Navarra, The Danish Research Ethics Committee System, Medical ethics committee of the Maastricht University, Ethics Committee of the Hotel-Dieu Hospital, University of Nottingham Medical School Ethics Committee, The Swedish National Council on Medical Ethics and Ethics Commission of the medical School of Toulouse.

Study Design and Subjects

The NUGENOB trial consisted in a 10-week dietary intervention with two types of hypo-energetic (~600 kcal/day) diets with a targeted fat energy of 20%–25% (low-fat diet, 15% energy from protein and 60–65 from carbohydrates) or 40%–45% (high-fat diet, with 15% energy from protein and 40–45 from carbohydrates) in obese patients [17]. The study involved 771 European obese adult subjects from eight recruiting centres of seven countries: United Kingdom (Nottingham), the Netherlands (Maastricht), France (Paris and Toulouse), Spain (Pamplona), Czech Republic (Prague), Sweden (Stockholm), and Denmark (Copenhagen). Obese patients participated in this randomised, parallel, two-arm, open-label multi-centre trial to assess weight loss. Obese patients were allocated to the two hypo-energetic diets by stratified block randomization using computer list generated at the coordinating centre.

The participants were 570 women and 192 men, aged 20–50 years and overweight or obese, with a high percentage (94.7%) of subjects with obesity, defined as having Body Mass Index (BMI) greater than or equal to 30 Kg/m² (Median BMI = 34.7 Kg/m²; Mean BMI = 35.6 Kg/m²; Standard deviation BMI = 4.9 Kg/m²). Exclusion criteria were: subjects with changes of more than 3 Kg within the three months previous to the trial, clinically diagnosed hypertension, diabetes or hyperlipidemia treated by drugs, untreated thyroid disease, surgically or drug-treated obesity, pregnancy, or alcohol or drug abuse.

Dietary Intervention

All the details regarding the dietary intervention are described in Petersen et al. [17] and in the web page http://www.nugenob.org.

Figure 1. Flow chart of the NUGENOB trial and genotyping success of MC3R variants. Legend to Figure 1: weight loss is expressed as mean ± standard deviation. doi:10.1371/journal.pone.0019934.g001
Before assignment to the dietary intervention group, all participants underwent a thorough standardized clinical and physiological examination described in Standard Operational Procedures applied in all participating centers. The target macronutrient composition of the two diets was: Low-fat (LF) diet (20%–25% of total energy from fat, 15% from protein and 60%–65% from carbohydrate) and High-fat (HF) diet (40%–45% of total energy from fat, 15% from protein, and 40%–45% from carbohydrate). Both diets were designed to provide 600 kcal/d fewer than the individually estimated energy requirements, which was calculated based on the estimation of the measured pre-treatment resting metabolic rate multiplied by a physical activity factor of 1.3. The dietary instructions were designed to minimize possible differences between the two diets in other components such as source and type of fat.

![Figure 2. MC3R polymorphisms genotyped in this study and linkage disequilibrium (r²) measures.](doi:10.1371/journal.pone.0019934.g002)

**Table 1. Basic statistical analysis of MC3R variants.**

<table>
<thead>
<tr>
<th>SNP</th>
<th>Position 20q</th>
<th>Minor Allele Frequency</th>
<th>Percentage of Genotyped</th>
<th>P-value HWE</th>
<th>P-value for differences among study centres</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs6024728</td>
<td>54252413</td>
<td>0.18</td>
<td>99.1</td>
<td>0.098</td>
<td>0.35</td>
</tr>
<tr>
<td>rs6014646</td>
<td>54253186</td>
<td>0.32</td>
<td>97.2</td>
<td>0.132</td>
<td>0.10</td>
</tr>
<tr>
<td>rs6024730</td>
<td>54254196</td>
<td>0.18</td>
<td>98.3</td>
<td>0.158</td>
<td>0.16</td>
</tr>
<tr>
<td>rs6024731</td>
<td>54254823</td>
<td>0.25</td>
<td>98.6</td>
<td>0.162</td>
<td>0.13</td>
</tr>
<tr>
<td>rs11697509</td>
<td>54256257</td>
<td>0.14</td>
<td>98.7</td>
<td>0.911</td>
<td>0.26</td>
</tr>
<tr>
<td>rs6127698</td>
<td>54256823</td>
<td>0.49</td>
<td>99.3</td>
<td>0.482</td>
<td>0.75</td>
</tr>
<tr>
<td>rs3746619</td>
<td>54257212</td>
<td>0.09</td>
<td>98.9</td>
<td>0.172</td>
<td>0.39</td>
</tr>
<tr>
<td>rs3827103</td>
<td>54257436</td>
<td>0.08</td>
<td>99.6</td>
<td>0.206</td>
<td>0.50</td>
</tr>
<tr>
<td>rs1543873</td>
<td>54261736</td>
<td>0.11</td>
<td>99.6</td>
<td>0.298</td>
<td>0.39</td>
</tr>
<tr>
<td>rs6099058</td>
<td>54263016</td>
<td>0.38</td>
<td>99.7</td>
<td>0.008</td>
<td>0.66</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0019934.t001
Statistical analyses

Descriptive summary statistics of the variables involved in the study was carried out, including estimation of genotype/allele frequencies and Hardy-Weinberg equilibrium (HWE) assessment. Disequilibrium coefficients $r^2$ and $D^*$ were computed as a measure of Linkage Disequilibrium (LD) between pairs of SNPs. Logistic regression analysis was used to assess the association between drop-out rates with genotypes and type of diet. Additional multivariate adjustment (BMI, age, sex and centre) yielded essentially the same conclusion as non-adjusted models.

The outcome variables assessed in this study were weight loss, fat-mass loss, waist circumference changes and REE changes by genotype after the intervention with hypo-caloric diets. Phenotype changes were calculated by subtracting measurements carried out immediately before randomization from measurements performed after the completion of the trial. Linear mixed models were used separately to assess changes for each outcome and for each SNP in $MC3R$ gene. Genotype codification was managed using the dominant model in which the genotypes were coded as 0, 1 or 2 depending on the number of alternative alleles in the genotype. Additionally, general models (genotypes as dummy variables with the homozygous wild-type as reference) as well as dominant and recessive models were fitted to the data. For weight loss, regression analyses were carried out with type of hypo-energetic diet, gender, age, age, age, age, age, age, age, centre and study (random variable effect) as covariates. In models for changes in fat mass, waist and REE, we controlled for respective baseline values, an interaction term between gender and initial values for each phenotype, as well as the type of hypo-energetic diet, gender, age, age, age, age, and centre of study (random variable effect). Changes in

| SNP                           | High-fat | Low-fat | Per-allele OR (95%CI) | P-value gene
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>rs6024728</td>
<td>CC</td>
<td>CA</td>
<td>1.1 (0.8–1.6)</td>
<td>0.69</td>
</tr>
<tr>
<td>rs6014646</td>
<td>AA</td>
<td>AT</td>
<td>1.1 (0.8–1.5)</td>
<td>0.10</td>
</tr>
<tr>
<td>rs6024730</td>
<td>GG</td>
<td>GA</td>
<td>1.3 (0.8–2.0)</td>
<td>0.92</td>
</tr>
<tr>
<td>rs6024731</td>
<td>AA</td>
<td>AG</td>
<td>1.1 (0.8–1.5)</td>
<td>0.36</td>
</tr>
<tr>
<td>rs11697509</td>
<td>CC</td>
<td>CG</td>
<td>1.3 (0.9–1.9)</td>
<td>0.36</td>
</tr>
<tr>
<td>rs6127698</td>
<td>GG</td>
<td>GT</td>
<td>0.8 (0.6–1.1)</td>
<td>0.31</td>
</tr>
<tr>
<td>rs3746619</td>
<td>CC</td>
<td>CA</td>
<td>1.3 (0.8–2.0)</td>
<td>0.92</td>
</tr>
<tr>
<td>rs3827103</td>
<td>GG</td>
<td>GA</td>
<td>1.4 (0.9–2.2)</td>
<td>0.77</td>
</tr>
<tr>
<td>rs1543873</td>
<td>TT</td>
<td>TG</td>
<td>1.3 (0.9–1.9)</td>
<td>0.92</td>
</tr>
<tr>
<td>rs6099058</td>
<td>GG</td>
<td>GA</td>
<td>1.2 (0.9–1.5)</td>
<td>0.54</td>
</tr>
</tbody>
</table>
| *Per-allele OR's for drop-outs and MC3R genotypes were calculated using logistic regression with the least frequent allele for each SNP coded a 0, 1 or 2 alleles (see statistical methods). doc:10.1371/journal.pone.0019934.0002
REE were additionally adjusted by fat-free mass and change in fat-free mass. Gene*diet interactions between MC3R genotypes and type of diet (low-fat and high-fat) were assessed in the context of co-dominant models for each outcome variable using the multivariate models described above. Haplotype-based association analysis was carried out for weight loss using likelihood ratio tests or permutations. Associations were adjusted by diet, initial weight, age, age$^2$, gender and centre of study as covariates. Statistical analyses were carried out with the programs R (v:2.8.1), STATA 11.0, HAPLOVIEW 4.1 and UNPHASED 3.1 [19].

As previously reported for statistical power calculations in the NUGENOB trial [20], the least detectable weight loss of main genetic effects ranged from 1.30–0.70 Kg for dominant models, from 7.72–0.99 Kg for recessive models, and from 1.25–0.55 Kg for additive co-dominant models (allele frequencies ranging from 0.05 to 0.5). Assuming an equal distribution of participants between the diets, the least detectable effects on weight loss of gene*diet interaction ranged from 2.49–1.07 Kg for additive co-dominant models. These calculations were performed with the assumptions of a statistical power of 0.80, a sample size of 642 individuals, and a mean weight loss of 6.8 Kg with a standard deviation of 3.5 Kg (software QUANTO 1.0; http://hydra.usc.edu/gxe).

## Results

Allele/genotype frequencies for MC3R polymorphisms and p-values for Hardy-Weinberg equilibrium are shown in Table 1. Very high levels of LD in our sample (Figure 2) were concordant with Hardy-Weinberg proportions except for rs6127698 (DOI: 0.47 and 0.43 respectively) to keep rs6099058 in the subsequent analyses. Statistical analyses were carried out with the programs R (v:2.8.1), STATA 11.0, HAPLOVIEW 4.1 and UNPHASED 3.1 [19].

As previously reported for statistical power calculations in the NUGENOB trial [20], the least detectable weight loss of main genetic effects ranged from 1.30–0.70 Kg for dominant models, from 7.72–0.99 Kg for recessive models, and from 1.25–0.55 Kg for additive co-dominant models (allele frequencies ranging from 0.05 to 0.5). Assuming an equal distribution of participants between the diets, the least detectable effects on weight loss of gene*diet interaction ranged from 2.49–1.07 Kg for additive co-dominant models. These calculations were performed with the assumptions of a statistical power of 0.80, a sample size of 642 individuals, and a mean weight loss of 6.8 Kg with a standard deviation of 3.5 Kg (software QUANTO 1.0; http://hydra.usc.edu/gxe).

Table 3. MC3R variants, weight loss and waist circumference change in the NUGENOB trial.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Per-allele* weight loss change (Kg)</th>
<th>Per-allele* waist change (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs6024728</td>
<td>0.23</td>
<td>0.30</td>
</tr>
<tr>
<td>rs6014646</td>
<td>-0.39</td>
<td>0.19</td>
</tr>
<tr>
<td>rs6024730</td>
<td>-0.24</td>
<td>0.23</td>
</tr>
<tr>
<td>rs6024731</td>
<td>-0.34</td>
<td>0.30</td>
</tr>
<tr>
<td>rs11697509</td>
<td>-0.27</td>
<td>0.27</td>
</tr>
<tr>
<td>rs6127698</td>
<td>0.25</td>
<td>0.18</td>
</tr>
<tr>
<td>rs3746619</td>
<td>-0.24</td>
<td>0.32</td>
</tr>
<tr>
<td>rs3827103</td>
<td>-0.03</td>
<td>0.34</td>
</tr>
<tr>
<td>rs1543873</td>
<td>-0.47</td>
<td>0.29</td>
</tr>
<tr>
<td>rs6099058</td>
<td>-0.15</td>
<td>0.18</td>
</tr>
</tbody>
</table>

*Per-allele changes were calculated using the number of the least frequent alleles for each SNP (coded a 0, 1 or 2 alleles) and under the co-dominant model adjusted for covariates (see statistical methods).

doi:10.1371/journal.pone.0019934.t003
Discussion

Genotype-dependent response to energy-restricted diets is a relevant topic in personalized nutrition [21], although the genetic effects of known genetic variants on weight loss induced by hypenergetic diets remain poorly defined [22]. In spite of the importance of the melanocortin system in energy balance [1,23–24], no information was available on the effect of MC3R on weight loss in obese adults submitted to hypo-energetic diets. In a study involving 184 obese children, Santoro et al. [16] found that rs3746619 (Thr6Lys) and rs3827103 (Val81Ile) of MC3R were associated with a differential weight loss in response to a negative energy balance in obese children (carriers of 6Lys and 81Ile alleles showed higher resistance to weight loss than non-carriers). We have also found in our study a trend for a greater resistance in losing weight in 6Lys and 81Ile carriers, although without achieving statistical significance. The missense variants Thr6Lys and Val81Ile of MC3R are in strong linkage disequilibrium which means that alleles 6Lys and 81Ile frequently occur in the same haplotype [10–14,16]. The simultaneous occurrence of alternative alleles in Thr6Lys and Val81Ile have been also associated in previous studies with childhood obesity, energy intake, eating behavior, substrate oxidation and first-phase insulin secretion [8,10–14,25].

Research in mice is concordant with a role for Mc3r acting as an inhibitory autoreceptor in Pomic neurons of the arcuate nucleus [26]. According to this role, it has been shown that peripheral administration of selective MC3R agonists stimulates feeding [23,26–28]. However, it is unlikely that this inhibitory action in the arcuate nucleus constitutes the predominant role of Mc3r in energy homeostasis given that this receptor is also expressed in multiple brain nuclei and peripheral tissues [29]. On the other hand, it has been also shown that Mc3r is implicated in the entrainment of food anticipatory behavior [30]. In addition to the

Figure 3. Crude weight loss in the NUGENOB trial by rs6014646, rs3746619 (Thr6Lys), rs3827103 (Val81Ile) and rs1543873 MC3R genotypes. Legend to Figure 3: P-values were calculated from multivariate models (see text). doi:10.1371/journal.pone.0019934.g003
possible effect of Mc3r on energy balance, there is also evidence in the literature supporting a different weight gain/loss pattern in Mc3r−/− mice according to the type of diet (high-fat versus low-fat). Butler et al. [28] described that the food intake of Mc3r−/− mice is not increased under low-fat or high-fat feeding in comparison with their wild-type littermates in the context of a specific genetic background. However, when Mc3r−/− mice on a B6 genetic background are fed a high-fat diet, they show hyperphagia and higher body fat when compared with the wild-type mouse [28]. On the other hand, a lower respiratory quotient was observed for Mc3r−/− mice fed with a low-fat diet compared to their wild-type littermates [28]. Finally, fatty acid oxidation in Mc3r−/− mice exposed to a low-fat diet seems to be reduced compared to the oxidation of wild-type mice [31].

Although the NUGENOB trial was primarily designed to study the effect of two diets on weight loss, a secondary goal was the assessment of genetic effects and interactions between diet and genes (NUGENOB stands for “Nutrition, genes and obesity”) [32]. Under this framework, the effect of 42 SNPs in 26 candidate genes were previously assessed in relation to weight loss, finding no significant associations after multiple testing adjustment [20]. Subsequently, an interaction effect was found between the diabetes-associated TCF7L genotypes and dietary fat/carbohydrate content [33], and also between FTO rs9999609 and macronutrient diet composition [34]. On the other hand, other studies indicated that a common variant genetic variation near MC3R gene (rs17782313), a direct candidate gene for food intake and obesity, does not seem to have a relevant effect on weight loss in adults after a lifestyle intervention [35].

Studies in monozygotic twins support the idea that weight loss after negative energy balance is under genetic control [36]. It is important to recognize that the study shown herein can be considered as exploratory, since multiple genetic and environmental factors may influence weight loss after hypo-energetic diets, making difficult the detection of consistent genotype-trait associations [22]. One reason why the main effects are so small could be due to the small magnitude of weight loss and because the study was based on mixed phenotypes with different genetic determinants, such as a combination of general level of obesity with dynamic changes over time. In this context, longitudinal twin studies with repeated assessment of BMI indicate that the genetic influences on general tracking level of BMI is different from those influencing the deviations from the general tracking level, suggesting that the genes influencing the tendency to become obese are different from those inducing individual differences in susceptibility to caloric restriction and dietary changes [37]. The difficulty in finding common genetic variation associated with complex traits is exemplified in the observational studies on increased BMI in large genome-wide epidemiological studies. In these studies common genetic variation seems to explain only a small proportion of BMI z-score in children or BMI in adults [15,38], with a limited predictive value for obesity risk [39–40]. It is conceivable that similar weak effects are present when assessing weight loss after hypo-energetic diets, even using intervention studies with moderately-large sample sizes such as the NUGENOB study.

Genetic variants of MC3R have been involved in obesity development mainly in relation with its role in the control of food intake [28]. Given that food intake is restricted in the intervention low-fat and high-fat groups, it was possible that the a priori hypothesized weight loss differences by MC3R genotype would have been arisen as a consequence of changes in energy expenditure. However, we have not found significant genetic effects on REE by MC3R genotype during the 10-week intervention trial. Regarding the analysis of food intake, it is also possible that assuming allocated diet (high-fat or low-fat) instead of measuring actual diet, has a disturbing effect in finding true associations, due to lack of compliance of participants with the energy deficit imposed by hypo-energetic diets. Regarding drop-out rates, weight loss intervention in the original analysis of the NUGENOB trial showed increased drop-out rates among participants randomized to high-fat compared to low-fat diet [17]. However, we have not found differences in drop-out by MC3R genotypes, suggesting that the assessment of the role of MC3R variants on weight loss is not affected by this factor. Finally, it is also important to mention that the assessment of gene*diet interactions was hampered by the increased sample size that would have been required to assess such effect in comparison with the sample size needed for evaluating main effects.

As a recapitulation, only MC3R rs6014464 achieved nominal statistical significance under the 0.05 level in relation to weight loss in adult obese subjects of the NUGENOB trial. However, this...
p-value cannot be considered as significant after strict control for multiple testing. Supporting the lack of effect of MC3R genotypes on anthropometric variables, tables 1 and 3 show point estimations for the genetic effects on weight loss, fat mass loss, waist and REE changes that are close to the zero effect, with relatively narrow confidence intervals. Moreover, no gene*diet interaction effects were found to be significant. In conclusion, the present study provided overall sufficient evidence to support that there is no major effect of genetic variants of MC3R and differential weight loss after a 10-week dietary intervention with hyper-energetic diets in obese Europeans.

Acknowledgments

The dietician core group and all other co-workers from the twelve participating centers are gratefully acknowledged. NUGENOB (“Nutrient-Genome interactions in human obesity-implications for dietary guidelines”) was supported by the European Community. Trial ISRCTN25367281 (Contract QLK1-CT-2000-00616). See www.nugenob.org for a complete list of NUGENOB contributors.

Author Contributions

Performed the experiments: AA OP WHMS IM JAM TIAS JMO. Analyzed the data: JLS RDJC CH AM KG CN TH. Contributed reagents/materials/analysis tools: OP TH CH. Wrote the paper: JLS TIAS JAM. Conceived and designed the NUGENOB study: TIAS AA OP WHMS.

References