Negative association of MC3R variants with weight and blood pressure in Cape Town pupils aged 11 - 16 years

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Background. Human and animal studies support the role of MC4R and MC3R in human obesity, but limited data are available on the genetic contribution to obesity in South African populations.

Objective. To screen obese-overweight South African pupils for MC3R and MC4R polymorphisms that may play a role in the development of obesity.

Design. A cross-sectional study screened 227 obese-overweight (115 black and 112 coloured) and 204 normal weight (94 black, 110 coloured) school pupils for the presence of MC4R and MC3R polymorphisms using a single strand conformation polymorphism, subsequent sequencing, and allele specific restriction enzyme analysis.

Results. Two polymorphisms were detected in the MC3R (T6K and V81I) but none in MC4R. After adjusting for age, gender and case-control status, the frequency distributions of T6K and V81I genotype and allele varied significantly between the ethnic groups. The frequency of the V81I A allele was significantly lower in coloured overweight-obesity than normal pupils. In coloured pupils, both polymorphisms were associated with obesity indices and total cholesterol. The T6K A allele was also associated with lower blood pressure. Likewise, different T6K-V81I haplotypes demonstrated negative associations with obesity indices and blood pressure.

Conclusion. We demonstrated that the MC3R polymorphisms have a protective effect on metabolic traits; however, further analysis is required to confirm whether this translates to a lower incidence of metabolic syndrome in coloured populations.

Obesity is a multifactorial body weight abnormality resulting from genetic and environmental interactions. Of the body mass index (BMI) variation among obese individuals of different populations and within the same family, 40 - 70% are estimated to be inherited, and 30% are attributed to cultural and socio-economic factors. More than 100 genes have been implicated in the heterogeneity of body weight, among which are genes that encode proteins or peptides of the central nervous system that affect conscious and unconscious aspects of energy intake and expenditure. Melanocortin receptors, particularly the melanocortin 4 receptor gene (MC4R), play a major role in the development of both monogenic and polygenic obesity. Mutations in MC4R are the most common cause of early onset obesity, accounting for up to 6%, particularly in white populations. Furthermore, more than 50 additional MC4R single nucleotide polymorphisms have been reported in obese and control individuals, with different effects depending on the population and family studied. Recently, obesity-related mutations (A299T, I335S, X361S, I183, A70T and M134I) have been identified in the melanocortin-3 receptor gene (MC3R) – another energy-regulating melanocortin receptor. Similar to MC4R polymorphisms, common variants in MC3R show significant frequency variation among different populations, accompanied by controversial effects on obesity phenotypes.

Epidemiological studies show that overweight and obesity prevalence rates in South African pupils are fast approaching those observed in developed countries, but the contribution of genetic factors is under-investigated. We analysed MC4R and MC3R for sequence variants that may contribute to the development of obesity in pupils from South Africa.

Methods

We aimed at determining the prevalence of metabolic syndrome and obesity in South African school pupils residing in Cape Town. A total of 431 (227 obese-overweight, and 204 normal) individuals of 11 - 16 years of age were selected, comprising black and coloured South Africans. The following measurements were performed on all pupils: anthropometric measurements (body weight and height, skinfold thickness), weight per square meter (kg/m2); and 3 readings were taken for blood pressure and waist and hip circumferences. Subjects were classified according to their weight status as obese, overweight and normal using the International Obesity Task Force criteria. Blood glucose, lipid levels and blood pressure have previously been described.

Mutation analysis and genotyping

Genomic DNA was extracted from either whole blood collected in vacutainer ethylenediamine tetra-acetic acid (EDTA) tubes or capillary blood collected onto Whatman FTA Cards (Merck Laboratories, UK). Subjects were screened for MCR3 and MCR4 coding sequence variants by amplifying the coding regions and splicing junctions and subsequent single strand conformation polymorphism (SSCP) analysis. Amplions with aberrant band patterns were further analysed by automated sequencing using ABI
3730. Subjects were genotyped for the presence of identified sequence variants using the allele-specific restriction enzyme analysis. The C allele of the K6T variant abolished the Mae III restriction enzyme recognition site, while the G allele of the V81I polymorphism created a second restriction recognition site for the BseD I enzyme.

Statistical analysis
Our sample size calculation was guided by the following factor: If the ‘risk’ allele frequency in the general population (controls) is 40%, then 227 cases and 204 controls have 80% power to detect, at a 5% significance level, a ‘risk’ allele frequency of 52% in cases.

Logistic regression assessed case-control association with genotype and race in joint models. Allelic distributions differed highly significantly between ethnic groups. The differences were such that adjusting trait-gene association analyses for ethnicity would not be valid. All analyses were therefore stratified by ethnicity. We tested association between quantitative traits and genotypes, alleles (additive) and haplotypes for the two polymorphisms. Triglycerides and diastolic and systolic blood pressure were log-transformed prior to analysis, because of skewed distributions. Effect estimates were obtained from linear models. Genotype-association (2 degrees of freedom) was tested by coding genotype as a categorical factor and allelic association (1 degree of freedom) with a numerical variable, counting the number of derived (A) alleles. We inferred haplotypes from the genotypes of the two variants and compared frequencies between obese and control learners. Models were adjusted for age and gender, and replicated measurements were controlled for as random effects.

Data were analysed using the programming language R (www.r-project.org) and specifically packages DGC-genetics (LD, Hardy-Weinberg, genotype and allelic association) and haplo.stats (inferred haplotype association).

Ethical approval was obtained from the Research Ethics Committee of the University of Stellenbosch (Project number: N07/07/160) and from the Faculty of Health and Wellness Science Ethics Committee, Cape Peninsula University of Technology (Project Reference number: CPUT/HAS-REC 0016). Written consent was obtained from the parents or guardians of pupils, and oral assent from pupils was obtained on the sampling day.

Results
Table I summarises physical characteristics and metabolic outcomes according to ethnicity. By selection, obesity indices were significantly higher in obese-overweight pupils. Analyses were adjusted for age and gender. The fasting blood glucose (FBG) was independently significantly higher in coloured than in black pupils (p = 0.0001) and obese-overweight than in normal-weight pupils (p = 0.0038). The HDL-C was higher in normal weight than obese-overweight pupils (p = 0.0008) and in black compared with coloured pupils (p = 0.0001). Triglyceride levels were similar in both racial groups, but significantly higher in obese-overweight pupils (p = 0.0038).

No sequence variants were identified on MC4R, while two common sequence variants (T6K and V81I) were identified in the MC3R coding region. The two coding variants were in strong linkage disequilibrium in all groups, ranging from D’ = 0.7437 in coloured normal-weight pupils to D’ = 0.9264 in the black obese pupils. The frequency distributions (both genotype and allele) differed significantly between the ethnic groups, after adjusting for case-control status. The differences were such that potential effects would be cancelled out if the genotype association test were adjusted for ethnicity. Therefore all subsequent analyses were stratified according to ethnicity. The frequency of the V81I A allele was significantly different between coloured overweight-obesity and normal weight pupils (respectively 28% and 40%, p = 0.0144) while that of the T6K A allele was only significantly different between the two ethnic groups. The A allele frequency was significantly higher in black pupils (p < 0.0001) (Table II). After adjusting for gender and age, both T6K and V81I alleles were only significantly different between obese-overweight and normal weight learners. Further, the allele frequencies of T6K and V81I did not differ between normal-weight learners in the proportion of cases who are ‘risk’ allele carriers is 52%.

Table I. Physical characteristics and metabolic outcomes of obese-overweight and normal black and coloured South African learners

<table>
<thead>
<tr>
<th>N</th>
<th>Black</th>
<th>Coloured</th>
<th>Black</th>
<th>Coloured</th>
<th>Obesity</th>
<th>Race</th>
</tr>
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<tbody>
<tr>
<td>115</td>
<td>112</td>
<td>94</td>
<td>110</td>
<td></td>
<td></td>
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<tr>
<td>Age (years)</td>
<td>13.2±2.0</td>
<td>13.4±2.0</td>
<td>13.2±2.0</td>
<td>13.6±2.1</td>
<td>0.3502</td>
<td>0.2674</td>
</tr>
<tr>
<td>Gender, male (%)</td>
<td>21 (25)</td>
<td>26 (31)</td>
<td>16 (19)</td>
<td>20 (24)</td>
<td>0.4208</td>
<td>0.4048</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>153.3±8.1</td>
<td>156±8.7</td>
<td>153.5±9.3</td>
<td>153.3±10.8</td>
<td>0.0983</td>
<td>0.1046</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.5±3.6</td>
<td>26.6±4.1</td>
<td>19.5±2.1</td>
<td>19.2±5.7</td>
<td>&lt;0.0001</td>
<td>0.5021</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>79.4±8.8</td>
<td>80.4±10.1</td>
<td>66.6±6.6</td>
<td>64.9±6.7</td>
<td>&lt;0.0001</td>
<td>0.8103</td>
</tr>
<tr>
<td>Hip (cm)</td>
<td>99.1±9.5</td>
<td>100.9±10.1</td>
<td>84.7±8.3</td>
<td>84.6±9.1</td>
<td>&lt;0.0001</td>
<td>0.2965</td>
</tr>
<tr>
<td>FBG (mmol/l)</td>
<td>3.9±0.8</td>
<td>4.2±0.8</td>
<td>3.8±0.8</td>
<td>4.1±0.7</td>
<td>0.0307</td>
<td>0.0001</td>
</tr>
<tr>
<td>TC (mmol/l)</td>
<td>3.8±1.0</td>
<td>3.7±0.8</td>
<td>3.7±0.8</td>
<td>3.7±0.8</td>
<td>0.5086</td>
<td>0.5539</td>
</tr>
<tr>
<td>TG (mmol/l)*</td>
<td>0.73 (0.57, 0.95)</td>
<td>0.65 (0.57, 0.99)</td>
<td>0.57 (0.57, 0.79)</td>
<td>0.57 (0.57, 0.80)</td>
<td>0.0038</td>
<td>0.2420</td>
</tr>
<tr>
<td>HDL-C (mmol/l)</td>
<td>1.1±0.4</td>
<td>0.9±0.3</td>
<td>1.2±0.4</td>
<td>1.1±0.4</td>
<td>&lt;0.0001</td>
<td>0.0008</td>
</tr>
<tr>
<td>SBP (mmHg)*</td>
<td>114 (104, 123)</td>
<td>111 (105, 117)</td>
<td>103 (97, 115)</td>
<td>104 (95, 116)</td>
<td>&lt;0.0001</td>
<td>0.5052</td>
</tr>
<tr>
<td>DBP (mmHg)*</td>
<td>69 (63, 78)</td>
<td>69 (64, 73)</td>
<td>64 (59, 71)</td>
<td>66 (59, 72)</td>
<td>&lt;0.0001</td>
<td>0.9976</td>
</tr>
</tbody>
</table>

Summary statistics: mean±SD unless indicated otherwise.
*p-values are for joint model, so each is adjusted for the other.
*Triglycerides and diastolic and systolic blood pressures were summarised as median (interquartile range) and were log-transformed to symmetry for tests.
BMI = body mass index; FBG = fasting blood glucose; TC = total cholesterol; TG = triglycerides; SBP = systolic blood pressure; DBP = diastolic blood pressure; HDL-C = high-density lipoprotein-cholesterol.
and V81I showed associations with obesity indices (body weight, BMI, hip and mid-upper arm) and total cholesterol, while only the T6K was associated with blood pressure in coloured pupils. In the latter pupils, the estimated effect of the T6K A allele was a decrease of 0.814 cm in mid-upper arm circumference ($p=0.0268$), 2.873 cm in hip circumference ($p=0.0099$), 3.225 kg in weight ($p=0.0268$) and a corresponding 0.959 kg/m$^2$ in BMI ($p=0.0213$). In addition, the T6K A allele reduced the systolic and diastolic blood pressures, respectively by 2.498 ($p=0.0467$) and 2.087 mmHg ($p=0.0362$), waist circumference by 2.141 cm ($p=0.0307$), hip circumference by 2.852 cm ($p=0.0071$), mid-upper arm circumference 0.878 cm ($p=0.0119$), weight 3.575 kg ($p=0.0074$) and BMI 1.168 kg/m$^2$ ($p=0.0110$).

There was no significant variation in the distribution of the T6K-V81I haplotype in the black group (global $p=0.1089$) between obese-overweight and normal weight pupils. Furthermore, no significant association between the T6K-V81I haplotype and physical or metabolic traits were observed in black pupils. However, the C-A was higher in normal weight (5% v. 2%), and A-A haplotype was higher in obese-overweight (52% versus 44%), respectively ($p=0.0688$) and 0.0749, while the C-G haplotype was most common in the normal weight (48%) rather than in obese-overweight (43%). In coloured pupils, the haplotype distributions differed significantly between obese-overweight and normal weight pupils (global $p=0.0185$); C-G was more prevalent in obese-overweight coloured pupils (69% v. 55%, $p=0.0063$) whereas C-A was more frequent in normal weight pupils (8% v. 2%, $p=0.0142$). In coloured pupils, after adjusting for age and gender, the C-A haplotype had 2.4 kg/m$^2$ less BMI than the C-G, while the A-A haplotype increased weight by 3.3 kg less. Systolic and diastolic blood pressures were decreased by 10 and 7.5 mmHg respectively in A-G haplotype compared with the C-G haplotype in coloured pupils.

### Discussion

Melanocortin 4 and 3 receptors are expressed in neurons of specific areas of the brain that regulate appetite and basal metabolic activity. Melanocortin receptors are part of the leptin-melanocortin pathway that plays a role in glucose homeostasis, insulin secretion, regulation of food intake, and energy balance. Neuropeptides in this pathway regulate food intake and energy balance by binding to melanocortin receptors, particularly MC4R, and to a lesser extent MC3R. Human and animal studies support the role of MC4R in human obesity, while MC3R variants have recently been implicated as pathogenic mutations that may cause dominantly inherited obesity. We analysed MC4R and MC3R for sequence variants that may contribute to polygenic obesity in two South African ethnic groups, namely coloured and black populations. Sequence analysis of MC3R in our study groups detected 2 common polymorphisms that showed variability in the distribution pattern between these groups.

The minor A allele frequency of both polymorphisms were higher in black pupils than in coloured, and almost equally distributed in the black population independent of obesity status. In the coloured group, the minor A allele of both polymorphisms in this study had a similar frequency ranging from 28% to 40% independent of obesity status. Compared with American whites, the homozygosity of these variants in coloured children was not as low. A prevalence of 15.8% of the T6K and V81I MC3R3 homozygote minor alleles in African-Americans compared with 1.7% in whites has been reported. The variants were associated with several metabolic traits in the coloured group. This association is further reflected in the inferred haplotype analysis. Both variants were associated with obesity indices and total cholesterol, but only T6K was associated with blood pressure in coloured pupils. In the black pupils, T6K was associated with triglyceride only.

Likewise, T6K-V81I haplotype analysis demonstrated a negative association of A-A and C-A haplotypes, compared with the C-G
A limitation of this study was the use of the SSCP method to detect possible sequence variants, which might have contributed to the detection rate. This technique has been reported to detect up to 98% (addition of 15% urea in both 8% and 10% polyacrylamide gels increases the detection limit from 90 to 98%) of nucleotide changes. SSCP is also subject to human error regarding interpretation of mobility shifts; and other sequence variants that could have been detected by ABI sequencing might have been missed.

Conclusion

Our results support the role of MC3R variants in weight regulation, with larger effects in the coloured population, in whom T6K and V81I polymorphisms also exact different metabolic effects, supporting the dual role of MC3R in regulating body weight and blood pressure. Although our results demonstrate that the MC3R polymorphisms have a protective effect on these metabolic traits, further study is required to confirm whether this translates to a lower incidence of metabolic syndrome in coloured populations.

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References