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ORIGINAL ARTICLE

Common variants near *MC4R* in relation to body fat, body fat distribution, metabolic traits and energy expenditure

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Objective: Common variants near *melanocortin receptor 4* (*MC4R*) have been related to fatness and type 2 diabetes. We examined the associations of rs17782313 and rs17700633 in relation to body fat, body fat distribution, metabolic traits, weight development and energy expenditure.
Methods: Obese young men ($n=753$, BMI $\geqslant 31.0\,kg\,m^{-2}$) and a randomly selected group ($n=874$) identified from a population of 174 800 men were re-examined in three surveys at mean ages 35, 46 and 49 years (S-35, S-46 and S-49). Measurements were available at upto eight times from birth to adulthood. Logistic regression analysis was used to assess odds ratio (OR) for the presence of the carrier allele for a given difference in phenotypic values.
Results: Rs17782313 minor C-allele was associated with overall, abdominal and peripheral fatness (range of OR = 1.06–1.14 per *z*-score units) at all three surveys, although only consistently significant at S-35 and S-46. Rs17700633 minor A-allele was also associated with the fatness measures, but significantly so only at S-49 for overall and abdominal fatness (range of OR = 1.03–1.15 per *z*-score units), and peripheral fatness (OR = 1.15–1.20 per *z*-score units). There were only few significant associations with metabolic traits. The rs17782313 C-allele and the rs17700633 A-allele were both associated with lower high-density lipoprotein cholesterol (range of OR = 0.64–0.84 per $mol\,l^{-1}$), significantly at S-46. The rs17700633 A-allele was significantly associated with insulin (OR = 1.25 per $50\,pmol\,l^{-1}$), leptin (OR = 1.42 per $10\,ng\,\mu l^{-1}$) and insulin sensitivity (OR = 0.81 per model unit). The rs17782313 C-allele and the rs17700633 A-allele were both associated with BMI in childhood and adolescence (range of OR = 1.04–1.17 per *z*-score units), significant for the rs17782313 C-allele at the age of 13–19 years and for rs17700633 A-allele at age 7, 10, 13 and 19 years. No significant associations were found for energy expenditure.
Conclusion: Near *MC4R* variants appear to contribute to body fat, body fat distribution, some metabolic traits, weight development during childhood, but not to energy expenditure.
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Introduction

Within the last couple of years new advanced gene identification strategies, especially the genome-wide association studies, have been the main contributors to success in the gene discoveries of common complex diseases, such as common obesity.[1,2] In the genetics of common obesity, the discovery of *FTO* was the first major success of genome-wide association studies, providing new insight into the pathogenesis of common obesity.[3–5] The *melanocortin receptor 4* (*MC4R*), located at chromosome 18q22, is involved in energy expenditure and appetite regulation,[6] and is a compelling candidate gene of obesity, as rare coding mutations consistently have been associated with monogenic obesity in humans.[7–9] In a large-scale genome-wide associa-

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tion studies, two novel common variants, mapping 188 kb (rs17782313; T/C) and 109 kb (rs17700633; G/A) downstream of *MC4R*, were found to associate with fatness in both adults and children.[10] The minor C-allele of rs17782313 and the minor A-allele of rs17700633, with a frequency of 0.24 and 0.30 respectively, and a low pair-wise linkage disequilibrium (LD) ($r^2 = 0.08$ in CEU HapMap), have modest allelic association with previously reported rare *MC4R* variants.[10] Thus, the associations between the novel common *MC4R* variant and fatness are unlikely to be explained by previously reported associations of rare *MC4R* variants with monogenic obesity, and it remains to be investigated if the near *MC4R* variants are involved in the MC4R pathway. Subsequent genome-wide association studies in individuals of Indian–Asian and European ancestry confirmed the associations between novel common variants near *MC4R* and fatness.[11] Albeit the identified variants were not identical, the most strongly associated variant (rs12970134) was in high LD with rs17782313 ($r^2 = 0.81$ in CEU HapMap).

In this study, we examined the associations of rs17782313, rs17700633 and rs12970134 near *MC4R* with (1) distinct specific fatness phenotypes and metabolic quantitative traits, (2) weight development throughout life, using repetitive assessments at upto eight times from birth to adulthood for the same individuals, and (3) energy expenditure.

Subjects and methods

Study population

Among 362 200 Caucasian men examined at the mean age of 20 years at the draft boards in Copenhagen and its surroundings during 1943–77, a randomly selected group of 1 in every 100 men ($n = 3601$) and all obese men ($n = 1930$) were manually identified. Obesity was defined as 35% overweight relative to a local standard in use at the time, and this corresponds to a BMI $\geqslant 31.0\,kg\,m^{-2}$, which proved to be above the 99th percentile. All obese and half of the random sample, still living in the region, were invited to a follow-up survey in 1982–1984 at the mean age of 35 years (survey S-35), in 1992–94 at the mean age of 46 years (survey S-46) and in 1998–2000 at the mean age of 49 years (survey S-49). The criteria for invitation to the follow-up surveys and the participation have been described previously,[12–14] and the number of participants shows the expected attrition over time (Table 1). Phenotypic assessments were carried out at all surveys, though much more thoroughly at survey S-49. DNA was sampled from buffy coats at S-46. In total, 1621 (747 obese and 874 randomly selected) participants were genotyped for this study, indicating that the randomly selected group represent 174 800 men, who were originally identified at the draft board examination. Among these 553 (232 obese and 321 randomly selected) had been assessed in S-49. From school health records, anthropometric measurements from birth and childhood (age 7, 10 and 13 years) were available[15] in a subset of the population and linked to the present cohort study. The Danish Data Protection Agency and the regional ethical committee approved the study to be in accordance with the Helsinki Declaration II. All participants signed a written consent before participating.

Phenotypic measurement

Waist circumference (cm) was measured according to the WHO recommendations to the nearest 0.5 cm with the subjects standing, using a nonexpendable linen tape measure,[16] Total body fat mass (kg) was assessed by bioimpedance at S-46 and from the DEXA scan at S-49. Fat body mass index (fat-BMI; $kg\,m^{-2}$) was calculated as total body fat mass (kg) divided by height (m) squared.

Table 1 Genotype distributions of rs17782313, rs17700633 and rs12970134 near *MC4R* for controls and obese participants in absolute numbers and percentages at survey S-35 ($n = 1325$), S-46 ($n = 1621$) and S-49 ($n = 553$)

	Controls				*Obese*			
rs17782313	*TT (%)*	*TC (%)*	*CC (%)*	*MAF*	*TT (%)*	*TC (%)*	*CC (%)*	*MAF*
S-35	424 (32.4)	286 (51.0)	30 (16.6)	0.23	281 (24.1)	247 (48.1)	44 (27.8)	0.29
S-46	502 (57.7)	328 (37.6)	41 (4.7)	0.24	362 (49.3)	312 (42.3)	62 (8.4)	0.30
S-49	186 (35.6)	117 (47.2)	16 (4.2)	0.23	117 (26.4)	92 (45.0)	21 (28.6)	0.29
rs17700633	*GG (%)*	*GA (%)*	*AA (%)*		*GG (%)*	*GA (%)*	*AA (%)*	
S-35	368 (32.4)	298 (51.0)	77 (16.6)	0.30	260 (24.1)	270 (48.1)	52 (27.8)	0.32
S-46	430 (49.3)	353 (40.3)	91 (10.4)	0.31	322 (43.2)	357 (47.7)	68 (9.1)	0.33
S-49	169 (35.6)	123 (47.2)	29 (17.2)	0.28	100 (26.4)	106 (45.0)	26 (28.6)	0.34
rs12970134	*GG (%)*	*GA (%)*	*AA (%)*		*GG (%)*	*GA (%)*	*AA (%)*	
S-35	388 (54.0)	292 (34.6)	38 (5.4)	0.26	252 (44.4)	263 (46.3)	53 (9.3)	0.33
S-46	454 (53.9)	339 (40.2)	50 (5.9)	0.26	322 (43.9)	337 (45.9)	75 (10.2)	0.33
S-49	176 (56.6)	117 (37.6)	18 (5.8)	0.25	100 (43.6)	108 (47.2)	21 (9.2)	0.33

Abbreviations: MAF, minor allele frequency; *MC4R*, *melanocortin receptor 4*. S-35, S-46 and S-49 denote the separate surveys in which participants were examined at the mean age of 35, 36 and 49 years, respectively.

Intra-abdominal adipose tissue (cm^2) was calculated from DEXA scans and anthropometry.[17] Lower body fat mass (%) was calculated from DEXA scans as the fat percentage of lower body fat mass.

Participants in S-35 and S-46 had non-fasting glucose and lipid levels (high-density lipoprotein (HDL)-cholesterol and triglycerides) determined on fresh plasma samples. In the S-49 cohort, an oral glucose tolerance test was conducted, except in individuals with diagnosed and, therefore, likely treated diabetes ($n = 10$).[12] Classification of impaired glucose tolerance (IGT) or type 2 diabetes was done according to the WHO diagnostic criteria.[18] Insulin sensitivity and acute insulin response were assessed by the recently recommended BIGTT indices (BIGTT-S_I and BIGTT-AIR, respectively) on the basis of measurements of plasma glucose and serum insulin at
time points 0, 30 and 120 min during the oral glucose tolerance test.[19] Details on data collections and measurement of anthropometric and other phenotypic estimates, including biochemical variables, have been described elsewhere.[12,20,21]

Molecular genetic analyses

Genotyping of *MC4R* rs17782313, rs17700633 and rs12970134 was carried out using Taqman allelic discrimination (KBioscience, Herts, UK). The genotype success rates were 97, 95.7 and 94.7%; error rates 0.28% in 721 duplicate samples, 0% in 196 duplicate samples and 0.18% in 551 duplicate samples for rs17700633, rs17782313 and rs12970134, respectively. All genotype groups obeyed Hardy–Weinberg equilibrium.

Statistical methods

A likelihood ratio test for an additive, a dominant and a recessive effect of the genotyped variants determined that a dominant genetic model was chosen for all three single-nucleotide polymorphisms (wild type versus heterozygous and homozygous genotype).

The data collected at each of the follow-up surveys, S-35, S-46 and S-49, were analyzed separately for each survey. The data on the obese and the controls were analyzed together by applying a logistic regression model. In this model, the probability of observing the particular genotype (presence of the minor allele) was defined as the dependent response variable and the quantitative values of the phenotypes irrespective of whether the individual originally belonged to the obese group or the control group. The massive enrichment of the right tail of the BMI distribution implies that the data cannot be analysed with BMI or BMI-associated outcomes conditional on the genotypes (for example by mean BMI per genotype) or as response variables in common regression models. Also, using a dichotomized case–control approach would waste considerable statistical efficiency otherwise gained by using quantitative phenotypes. Hence, to take advantage of the greater statistical power and much wider coverage of the phenotypes, we reversed the statistical models for the associations and examined the probability of carrying the particular risk-allele genotype for a given level of the phenotypes. This can be done without distributional assumptions about the phenotypes. Thus, logistic regression analysis was used to assess the odds ratios (ORs) of the genotype (response variable) in relation to the phenotypes (covariates) in the combined case and control groups with and without adjustment for the concurrent fat-BMI ($kg\,m^{-2}$). Information for fat-BMI was not available in S-35, and therefore we adjusted for BMI in these analyses.

To obtain similar fatness units, all anthropometrics and body composition measures were converted to age-adjusted *z*-scores that indicate the deviations from the population mean values in s.d. units. The age-adjusted *z*-scores were calculated from the mean values and s.d. of the randomly selected control group and applied to the entire study population. In other words, 1 *z*-score unit (U) is equal to 1 s.d. of the particular obesity phenotype in the control sample. In the remainder analyses, age at examination was included as a continuous covariate. The OR for being carrier of the minor allele according to BMI *z*-score should be interpreted as an increment in odds for being carrier of the minor allele per U increase in BMI *z*-score. Nominal *P*-values are reported, and the interpretation of them in the light of multiple testing is included in the Discussion section. Analyses were carried out using SAS statistical procedures (version 9.1; SAS Institute Inc, Cary, NC, USA).

Results

The minor allele frequencies of rs17782313, rs17700633 and rs12970134 near *MC4R* were almost exactly the same at the three surveys indicating that the attrition of the study groups by time was not dependent on the genotype (Table 1). In controls, the minor allele frequencies of rs17782313, rs17700633 and rs12970134 were 0.23–0.24, 0.28–0.31 and 0.25–0.26, respectively, almost analogous to the reported distribution in European Caucasians.[10] Rs17782313 and rs17700633 were in low LD with each other ($r^2 = 0.13$), rs17782313 was in high LD with rs12970134 ($r^2 = 0.75$), whereas rs17700633 was in low LD with rs12970134 ($r^2 = 0.18$) in the present study population. All results obtained for rs12970134 were identical to those obtained for rs17782313, and are therefore not reported here.

Overall, abdominal and peripheral fatness

We estimated the ORs of the genotype (response variable) in relation to the phenotypes (covariates). Results from logistic regression analyses for fatness phenotypes are given in ORs with 95% confidence intervals.

All obesity-related phenotypes elucidating overall, abdominal and peripheral fatness were associated with rs17782313

Table 2 Odds ratio (OR) including 95% confidence intervals (CI) for *MC4R* rs17782313 C-allele carriers and for rs17700633 A-allele carriers in relation to fatness phenotypes (in age-adjusted *z*-scores)

	S-35 N = *1325* *OR (95% CI)*	P	*S-46* N = *1621* *OR (95% CI)*	P	*S-49* N = *553* *OR (95% CI)*	P
Rs17782313						
FBMI (kg m^{-2})	—	—	1.08 (1.01; 1.15)	0.022	1.07 (0.97; 1.18)	0.207
BMI (kg m^{-2})	1.06 (1.00; 1.12)	0.037	1.07 (1.00; 1.13)	0.038	1.07 (0.97; 1.17)	0.172
Waist (cm)	—	—	1.08 (1.01; 1.15)	0.025	1.10 (0.99; 1.22)	0.083
IAAT (cm^2)	—	—	—	—	1.07 (0.96; 1.20)	0.243
Hip (cm)	—	—	1.07 (1.00; 1.15)	0.039	1.11 (1.01; 1.22)	0.039
LBFM (%)	—	—	—	—	1.14 (1.00; 1.28)	0.042
Rs17700633						
FBMI (kg m^{-2})	—	—	1.04 (0.98; 1.11)	0.196	1.15 (1.04; 1.27)	0.007
BMI (kg m^{-2})	1.03 (0.98; 1.09)	0.257	1.04 (0.98; 1.10)	0.244	1.14 (1.03; 1.25)	0.008
Waist (cm)	—	—	1.05 (0.99; 1.13)	0.121	1.16 (1.04; 1.29)	0.006
IAAT (cm^2)	—	—	—	—	1.14 (1.02; 1.28)	0.022
Hip (cm)	—	—	1.04 (0.98; 1.11)	0.231	1.15 (1.05; 1.27)	0.004
LBFM (%)	—	—	—	—	1.20 (1.06; 1.36)	0.004

Abbreviations: BMI, body mass index; FBMI, fat body mass index; IAAT, intra-abdominal adipose tissue; LBFM, lower body fat mass. S-35, S-46 and S-49 denote the separate surveys, in which participants were examined at the mean age of 35, 36 and 49 years, respectively. Within each survey, the obese and the control group were analysed together by using the phenotype, for example BMI, as covariate in a logistic regression model with odds of the genotype as the response variable.

at survey S-35 and S-46 (Table 2), where a 1 U *z*-score increase in obesity phenotypes increased the odds for the minor allele carriers by 6–8% (OR = 1.06–1.08, $P = 0.022$–0.039). Significant associations were observed in relation to hip circumference and lower body fat mass (%) at survey S-49, where a 1-U increase in hip circumference and lower body fat mass increased the odds for the C-allele carriers by 11–14% (OR = 1.11–1.14, $P = 0.039$–0.042). For overall and abdominal fatness at S-49, estimates were similar to the obtained estimates at S-46, but did not reach statistical significance.

Compared with rs17782313, we obtained stronger significant associations between the examined obesity phenotypes and rs17700633, albeit only at S-49 (Table 2). A 1-U increase in overall and abdominal fatness increased the odds for the A-allele by 14–16% (OR = 1.14–1.16, $P = 0.006$–0.022). For peripheral fatness, a 1-U *z*-score increase in hip circumference and lower body fat mass (%) increased the odds for the A-allele by 15–20% (OR = 1.15–1.20, $P = 0.004$).

The associations between the near *MC4R* variants and all body fat measures were weakened when adjusted for fat-BMI, and neither of these measures nor fat-BMI itself maintained statistical significance when analysed together (data not shown).

Metabolic traits

Generally, there were only few statistically significant results (Table 3). Thus, none of the associations between the *MC4R* variants and circulating levels of glucose, C-peptide, HbA1C, triglyceride, free fatty acids, fibrinogen, ASAT or blood pressure reached statistical significance.

Both variants near *MC4R* were associated with serum HDL-cholesterol levels at S-46, where a 1-U increase in mmol l^{-1} HDL-cholesterol decreased the odds for the rs17782313 C-allele and rs17700633 A-allele by 24 and 28%, respectively (OR = 0.76, $P = 0.03$ and OR = 0.72, $P = 0.008$, respectively). Similar estimates were obtained at survey S-35 and S-49, but they did not reach statistical significance.

At S-49, an association between plasma insulin and rs17700633 was observed, where each 50-pmol l^{-1} increase of insulin increased the odds for the A-allele by 25% (OR = 1.25, $P = 0.03$) (Table 3). An association between a surrogate measure of insulin sensitivity and rs17700633 was obtained. A 1-U increase in BIGTT-S_I index decreased the odds for the A-allele by 19% (OR = 0.81, $P = 0.038$).

A strong significant association between circulating leptin levels and rs17700633 was obtained at S-49, where a 10-U increase in circulating leptin increased the odds for the A-allele by 42% (OR = 1.42, $P = 0.005$).

As expected, the significant associations were weakened when adjusted for fat-BMI (data not shown).

Childhood and adolescence obesity

Except at birth, associations were observed between both near *MC4R* variants and BMI *z*-score during childhood and adolescence (Table 4). A 1-U increase in BMI *z*-score increased the odds for the rs17782313 C-allele by 12 and 7%, respectively at age 13 and 19 years (OR = 1.12, $P = 0.034$ and OR = 1.07, $P = 0.0004$, respectively). Associations for age 7 and 10 years showed similar estimates, but were not significant. A 1-U increase in BMI *z*-score increased the odds for the rs17700633 A-allele by 15, 17, 17 and 4%, respectively at age 7, 10, 13 and 19 years (OR = 1.15, $P = 0.015$; OR = 1.17, $P = 0.005$, OR = 1.17, $P = 0.005$ and OR = 1.04, $P = 0.038$, respectively).

Table 3 Odds ratio (OR) including 95% confidence intervals (CI) for *MC4R* rs17782313 C-allele carriers and for rs17700633 A-allele carriers in relation to metabolic traits and OGTT-derived indices

	S-35 N = *1291* *OR (95% CI)*	P	*S-46* N = *1607* *OR (95% CI)*	P	*S-49*[a] N = *547* *OR (95% CI)*	P
Rs17782313						
Metabolic traits						
p-Glucose (10 mmol l^{-1})	1.06 (0.48; 2.35)	0.88	1.03 (0.98; 1.05)	0.38	0.99 (0.29; 0.36)	0.98
s-Insulin (50 pmol l^{-1})	—	—	—	—	1.16 (0.96; 1.41)	0.13
s-HDL (mmol l^{-1})	0.74 (0.48; 1.13)	0.16	0.76 (0.60; 0.97)	0.03	0.73 (0.41; 1.32)	0.30
s-Triglycerides (10 mmol l^{-1})	—	—	—	—	1.89 (0.45; 7.95)	0.39
Systolic BP (10 mm Hg)	1.02 (0.95; 1.09)	0.61	0.99 (0.93; 1.04)	0.63	0.94 (0.99; 1.01)	0.95
Diastolic BP (10 mm Hg)	1.02 (0.93; 1.11)	0.72	1.01 (0.92; 1.10)	0.91	0.95 (0.85; 1.04)	0.21
s-C-peptide (500 mmol/l)	—	—	—	—	1.08 (0.85; 1.37)	0.51
HbA1C (%)	—	—	—	—	1.90 (0.20; 18.1)	0.58
FFA (mmol l^{-1})	—	—	—	—	1.42 (0.48; 4.26)	0.52
Fibrinogen (g l^{-1})	—	—	1.00 (0.99; 1.02)	0.54	—	—
ASAT (IU l^{-1})	—	—	0.66 (0.37; 1.19)	0.17	—	—
Leptin (ng μl^{-1})					1.17 (0.94; 1.47)	0.17
Derived indices						
BIGTT-S_I	—	—	—	—	0.89 (0.73; 1.07)	0.21
BIGTT-AIR	—	—	—	—	1.17 (0.89; 1.54)	0.25
Rs17700633						
Metabolic traits						
p-Glucose (10 mmol l^{-1})	0.64 (0.29; 1.43)	0.27	1.01 (0.98; 1.05)	0.54	1.22 (0.36; 4.16)	0.75
s-Insulin (50 pmol l^{-1})	—	—	—	—	1.25 (1.02; 1.54)	0.030
s-HDL (mmol l^{-1})	0.84 (0.55; 1.28)	0.41	0.72 (0.57; 0.92)	0.008	0.64 (0.35; 1.14)	0.13
s-Triglycerides (10 mmol l^{-1})	—	—	—	—	1.83 (0.43; 7.84)	0.41
Systolic BP (10 mm Hg)	0.99 (0.92; 1.07)	0.83	1.05 (0.99; 1.11)	0.10	0.99 (0.89; 1.09)	0.76
Diastolic BP (10 mm Hg)	1.01 (0.92; 1.10)	0.92	1.05 (0.96; 1.14)	0.27	0.97 (0.83; 1.13)	0.68
s-C-peptide (500 mmol l^{-1})	—	—	—	—	1.25 (0.98; 1.58)	0.07
HbA1C (%)	—	—	—	—	1.89 (0.20; 18.3)	0.58
FFA (mmol l^{-1})	—	—	—	—	0.46 (0.16; 1.37)	0.16
Fibrinogen (g l^{-1})	—	—	1.00 (0.98; 1.01)	0.47	—	—
ASAT (IU l^{-1})	—	—	1.08 (0.62; 1.89)	0.79	—	—
Leptin (10 ng μl^{-1})					1.42 (1.12; 1.82)	0.005
Derived indices						
BIGTT-S_I	—	—	—	—	0.81 (0.67; 0.99)	0.038
BIGTT-AIR		—	—	—	1.27 (0.96; 1.66)	0.09

Abbreviations: BIGTT-AIR, OGTT-derived index of acute insulin response; BIGTT-S_I, OGTT-derived index of insulin sensitivity; BP, blood pressure; HbA1C, glycated hemoglobin; HDL, high-density lipoprotein; p, plasma; s, serum. S-35, S-46 and S-49 denote the separate surveys in which participants were examined at the mean age of 35, 36 and 49 years, respectively. S-35, S-46 and S-49 denote the separate surveys, in which participants were examined at the mean age of 35, 36 and 49 years, respectively. Within each survey, the obese and the control group were analysed together by using the phenotype, for example body mass index (BMI), as covariate in a logistic regression model with odds of the genotype as the response variable. All analyses were adjusted for age. [a]All values for the S-49 are derived from the oral glucose tolerance test (OGTT) examination, and are therefore fasting compared to non-fasting for S-35 and S-46. The units of the covariates were chosen to make the odds ratios readable with significant digits even though this results in unrealistic unit values.

Table 4 Odds ratio (OR) including 95% confidence intervals (CI) for *MC4R* rs17782313 C-allele carriers and for rs17700633 A-allele carriers in relation to birth weight and age-adjusted BMI *z*-scores at childhood and adolescence

	Rs17782313 *OR (95% CI)*	P	*Rs17700633* *OR (95% CI)*	P
Birth weight, $n = 329$	1.01 (0.82; 1.23)	0.95	1.04 (0.85; 1.27)	0.69
BMI z-score of:				
7 years, $n = 382$	1.08 (0.98; 1.20)	0.14	1.15 (1.03; 1.28)	0.015
10 years, $n = 381$	1.11 (0.99; 1.23)	0.07	1.17 (1.05; 1.30)	0.005
13 years, $n = 373$	1.12 (1.01; 1.25)	0.04	1.17 (1.05; 1.30)	0.005
19 years, $n = 1\ 621$	1.07 (1.03; 1.11)	0.0004	1.04 (1.00; 1.08)	0.038

Abbreviation: BMI, body mass index. Within each age, the obese and the control group were analysed together by using the phenotype, e.g. BMI, as covariate in a logistic regression model with odds of the genotype as the response variable.

Table 5 Odds ratio (OR) including 95% confidence intervals (CI) for *MC4R* rs17782313 C-allele carriers and for rs17700633 A-allele carriers. In relation to resting energy expenditure ($n=547$), LTPA ($n=541$), VO_2max ($n=463$) and glucose-induced thermogenesis ($n=535$)

	S-49 OR (95% CI)	P
Rs17782313		
Resting energy expenditure (MJ)	1.37 (0.82; 2.29)	0.23
LTPA		
<2 h light PA per week	1.00	0.78[a]
2–4 light PA per week	1.25 (0.70; 2.25)	0.45
>4 h light or 2–4 h moderate PA per week	1.05 (0.57; 1.95)	0.77
>4 h moderate PA per week	1.15 (0.42; 3.10)	0.92
VO_2max (l min^{-1})	1.07 (0.76; 1.50)	0.70
Glucose-induced thermogenesis	1.05 (0.90; 1.21)	0.55
Rs17700633		
Resting energy expenditure (MJ)	1.24 (0.74; 2.07)	0.41
LTPA		
<2 h light PA per week	1.00	0.63[a]
2–4 light PA per week	0.92 (0.52; 1.64)	0.40
>4 h light or 2–4 h moderate PA per week	1.16 (0.63; 2.13)	0.56
>4 h moderate PA per week	1.14 (0.42; 3.08)	0.80
VO_2max (l min^{-1})	1.03 (0.73; 1.44)	0.87
Glucose-induced thermogenesis (MJ)	1.05 (0.91; 1.22)	0.53

Abbreviations: MJ, mega Joule; l, liter; LTPA, leisure-time physical activity; MJ, mega Joule. S-49 denotes the survey in which participants were examined at the mean age of 49 years. The obese and the control group were analysed together by using the phenotype, for example body mass index (BMI), as covariate in a logistic regression model with odds of the genotype as the response variable. All analyses were adjusted for age. [a]*P* for monotonic trend.

Energy expenditure

None of the two variants near *MC4R* were significantly associated with resting energy expenditure, leisure-time physical activity, VO_2max or glucose-induced thermogenesis (Table 5).

Discussion

The present case–cohort study of Danish men assessed at upto eight points in time confirmed associations of rs17782313, rs17700633 and rs12970134 near *MC4R* with human fatness from childhood through middle age. As rs12970134 was in high LD with rs17782313 ($r^2=0.75$), all results were identical for these two single-nucleotide polymorphisms. Carriers of the rs17782313 C-allele were more frequently observed the greater the BMI and the larger the waist circumference than carriers homozygous for the major allele. Similar associations were observed for carriers of the rs17700633 A-allele compared with carriers homozygous for the major allele. Although previous studies have suggested additive effects of the minor alleles, our data suggested that a dominant pattern fitted better to the data, which may be due to the manifold enrichment of the sample in the upper tail of the BMI distribution. Except for associations for rs17700633 with circulating HDL-cholesterol, leptin and insulin and with a surrogate measure of insulin sensitivity (BIGTT-S_I), all most likely explained by effect of fat-BMI, there were no consistent and significant relations to metabolic traits.

The rs17782313 C-allele near *MC4R* was associated with overall (BMI, fat-BMI), abdominal (waist circumference, intra-abdominal adipose tissue) and peripheral (hip circumference, lower body fat mass) fatness. The strength of the associations between the SNPs and the various fatness phenotypes was about equal throughout the range of each phenotype at survey S-46 and S-49 and comparable to previously reported studies.[10] Although estimates were similar, the results from S-49 did not reach statistical significance, except for peripheral fatness. Compared with rs17782313, stronger significant associations were obtained at S-49 between the A-allele of rs17700633 near *MC4R* and overall, abdominal and peripheral fatness, but not at the preceding surveys. All the associations were weakened when adjusted for fat-BMI and became statistically non-significant. To partial out any fat-BMI independent effects of the near *MC4R* variants on the various body fat distribution measures would require greater statistical power.

Associations were observed between rs17782313 and HDL-cholesterol levels, and between *MC4R* rs17700633, plasma insulin, serum HDL-cholesterol, circulating leptin and insulin sensitivity. As expected, adjusting for fat-BMI weakened these associations. However, as for body fat distribution, greater statistical power is needed to convincingly discern fat-BMI independent effects. The results are analogous to the association of a common *FTO* variant (rs9939609) with type 2 diabetes.[3–5,22] However, in our study, associations with plasma insulin and insulin sensitivity were obtained for rs17700633, which is in contrast to other studies that report associations between insulin measures and rs17782313 and/or rs12970134.[11,23,24] The mediating effect of fat-BMI on metabolic traits and type 2 diabetes seem to be less clear across different populations.[10,11,24] It is to be noted that except for associations with HDL-cholesterol, insulin, insulin-sensitivity and leptin, none of the other obesity-associated metabolic traits were significantly associated with the near *MC4R* variants.

We observed stronger associations for rs17700633 than with rs17782313, which is not in line with previous studies.[10,11,24] Rs17782313 near *MC4R* has been reported to be the most significantly associated variant,[10] and most studies do not report significant associations for rs17700633.[10,11,24] One recent study, however, has reported associations between rs17700633 and obesity phenotypes, but not with metabolic traits.[23] In the same study, the results for rs17782313 were abolished when the effect of rs12970134 was taken into account, whereas rs12970134 and rs17700633 seemed to have independent effects, which is analogous to our results. A potential explanation is that rs17700633 near

MC4R may have a more pronounced effect on extreme obesity. This means, that studies, in which the extreme ranges of the obesity phenotypes are not represented, may not be able to detect such an association.

Analysis from the school health records of the participants revealed that the rs17782313 C-allele *MC4R* was associated with BMI *z*-score in childhood and adolescence, though only statistically significant at ages 13 and 19 years. The *MC4R* rs17700633 A-allele was associated with BMI *z*-score in both childhood and adolescence (age 7, 10, 13 and 19 years). Combined with the results obtained during adulthood, it appears that these variants have a considerable impact on body weight from childhood though adulthood. No associations were obtained for birth weight, which is in agreement with a previously reported study.[10] As rare severe coding mutations in *MC4R* are associated with increases in lean body mass, bone mineral density and enhanced linear growth,[25] it may be of interest to examine associations with body weight changes and linear growth in a future study.

The examined variants were in our study not associated with resting energy expenditure, leisure-time physical activity, VO_2max or glucose-induced thermogenesis. These results suggest that the examined variants may not be associated with energy expenditure. Rs17782313 near *MC4R* has recently been associated with higher energy and fat intake.[24] Experimental data show that *MC4R* may have a role in controlling fat intake preferences,[26] but the mechanisms underlying these observations need to be further elucidated.

The strengths of the present population-based study of Danish Caucasian men include the sampling design, wherein the controls were randomly selected from the same population in which the cases were identified and followed up for approximately 30 years. Repetitive detailed assessment of anthropometric and physiological variables at mean ages 35, 46 and 49 years, and the possibility of linking data on BMI from school health records for the same individuals makes the present study population unique and appropriate for investigating the impact of gene variants on body weight development. In addition, fatness was examined through a broad range of fatness and at the age of inclusion, the obese participants represented the most extreme range of the fatness phenotypes, above the 99th percentile.

However, several limitations need to be acknowledged. The sampling design does not allow the usual simple estimation of distribution parameters for the phenotypes for given genotypes, for example mean BMI per genotype. Population stratification may occur because of systematic admixture of ancestry, and may lead to spurious associations.[27] However, population stratification is unlikely to explain the obtained results because of the homogenous study population of Danish Caucasian men, in which the examined genotype distributions complied with Hardy–Weinberg equilibrium. The observed effect sizes may seem relatively small, and may be too small to disentangle fat-BMI dependent and independent components. However, the effects may still have impact on health; in a recent large population-based study, a surprisingly little increase in body weight throughout the range of body weights was associated with increases in risk of coronary heart disease.[28] As the findings of this study are restricted to men, the findings need to be replicated in other populations. Potential sex-related effects have been suggested and results from a study suggest that their obtained association between rs17782313 *MC4R* and type 2 diabetes may be more evident in women.[24] The examined genotypes show almost similar distribution for the minor allele frequencies and, hence, argue against that the attrition of the samples from the three surveys is related to the particular examined associations. The present sample size may seem small for a genetic association study, and especially the threefold drop in sample size from S-46 to S-49 may have reduced the statistical power. Precision of the measurement may also reduce the statistical power, for example measurements of abdominal obesity and amount of visceral fat, although usable,[29] might have been much better using more advanced imaging technology, such as computed tomography scanning and nuclear magnetic resonance imaging. On the other hand, keeping the phenotypes as quantitative variables in the analyses, the efficiency is considerably higher than in usual case–control type of analysis, as reflected in the fairly narrow confidence intervals; this means that we thereby have narrowed down the likely true OR's that could have given rise to the observed OR's. The number of statistical analyses carried out invites concerns about the possible implications of multiple testing for the interpretation of the nominal *P*-values. Valid adjustment for the multiple testing may not be feasible because of lack of clear delineation of tests belonging to the same test series, but caution should be exercised in the interpretation based on *P*-values, and major emphasis put on the consistency of the results, both internally and in comparison with previous studies.

In conclusion, the results from this study confirmed that rs17782313, rs17700633 and rs12970134, near *MC4R*, contribute to individual differences in human fatness during childhood and adulthood, but not to birth weight. The A-allele of rs17700633 exhibited somewhat stronger associations than the C-allele of rs17782313 or the A-allele of rs12970134, the latter two showing the same associations. Only few metabolic traits (HDL-cholesterol, insulin, insulin sensitivity and leptin) were associated with these variants, and these associations were most likely explained by the association with fatness.

Conflict of interest

The authors declare no conflict of interest.

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