

ARTICLE

# Proopiomelanocortin gene variants are associated with serum leptin and body fat in a normal female population

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A major quantitative trait locus (QTL) determining leptin levels has been linked to the proopiomelanocortin (*POMC*) region on chromosome 2. Most studies, based on under 350 lean or obese subjects, have shown no association between *POMC* SNP 8246 C/T and serum leptin, but significant associations have been reported with *RsaI* 8246 C/T SNP haplotypes. We have investigated association of four *POMC* SNPs with body composition and serum leptin in 2758 normal Caucasian female subjects (mean age  $47.4 \pm 12.5$  years), from the St Thomas' UK Adult Twin Registry (Twins UK): *RsaI* and 51 G/C in the 5'UTR and 8246 C/T and 7965 C/T in the 3'UTR. Under the recessive model, the 8246 T allele (freq. 0.18) was significantly associated with higher mean BMI ( $P=0.032$ ) and total fat ( $P=0.046$ , both after age adjustment). Significant associations were maintained in sib-TDT with waist ( $P=0.049$ ), total fat ( $P=0.037$ ) and emerged with serum leptin ( $P=0.016$ ). Initial significant associations between *RsaI* (-) allele (freq. 0.30) and higher waist ( $P=0.04$ ) or % central fat ( $P=0.02$ ) were not maintained in sib-TDT. No significant associations were found between body composition or serum leptin and *RsaI*/8246 C/T haplotype and none with 51 G/C (freq. 0.01) or 7965 C/T (freq. 0.004). There was minimal pairwise LD between the four loci, apart from *RsaI* and 8246 C/T ( $D' = -0.78$  ( $P < 0.0001$ )). Associations of BMI, weight and total fat with SNPs in regions flanking the *POMC* gene in this powerful study suggest that regulation of *POMC* expression may be influential in determining body weight.

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## Introduction

The hormone leptin is a satiety factor secreted predominantly by adipose tissue, which binds to receptors in the hypothalamus<sup>1</sup>, invoking depression of appetite, suppres-

sion of lipogenesis and increased oxidation of stored fat.<sup>1</sup> Proopiomelanocortin (*POMC*) is expressed in response to binding of leptin to cognate receptors on a distinct population of neurons in the arcuate nucleus.<sup>2</sup> *POMC* is initially synthesised as a large precursor, which undergoes limited proteolysis to several smaller peptides,<sup>3</sup> including  $\alpha$ -melanocortin-stimulating hormone ( $\alpha$ -MSH),<sup>5</sup> the natural ligand of the melanocortin-4 receptor (*MC4R*).<sup>4</sup> Activation of the *MC4R* elicits downstream events, which effect the anorexigenic response to leptin.<sup>5</sup>

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The *POMC* gene maps to chromosome 2p23.3.<sup>6</sup> A major quantitative trait locus (QTL) determining leptin levels has been linked to the *POMC* region in Mexican American families<sup>7</sup> and replicated in two other studies,<sup>8,9</sup> suggesting that variation in body mass index (BMI) or fat mass in the population might be linked to functional variant(s) in *POMC* coding or regulatory sequences.

Several studies have examined the common *POMC* SNP in exon 5 3'UTR, 8246 C/T (GenBank Accession V10510 nt 8246; NT 022184 nt 4199820; dbSNP rs1042571). (This SNP appears in early papers as 7566 C/T on a different numbering system.<sup>10</sup>) So far, no association has been found with obesity in case-control studies<sup>11-13</sup> and small-scale studies (less than 350 subjects) have mainly failed to detect an association with BMI, serum leptin or body composition in either obese or nonobese subjects.<sup>11,12</sup> However, recently Suviolahti *et al*<sup>13</sup> in lean, but not in obese subjects, have found an association of the C allele with higher serum leptin ( $P=0.003$ ). In this mixed sample, the female subjects were the main contributors to significance ( $P=0.004$ ) and the association was even more pronounced in lean female carriers of 8246 CC/*RsaI* (–/–) or (+/–) genotype combination ( $P<0.0005$ ). *RsaI*, discovered by Feder *et al*<sup>14</sup> and located by Suviolahti *et al*,<sup>13</sup> lies 1802 bp upstream of the transcription start site (GenBank Accession NT 022184 nt 4209187 G/A; dbSNP rs3754860). Hixson *et al*<sup>15</sup> had previously reported a different *POMC RsaI/8246C/T* genotype combination (8246 TT/*RsaI* (+/+)) associated with higher serum leptin levels ( $P=0.001$ ) in obese Mexican Americans.

The striking association of the 8246 C/T SNP with serum leptin in normal Caucasian subjects discovered by Suviolahti *et al*<sup>13</sup> demands replication, as it had been observed in only 70 female subjects and others had been unable to establish associations in, respectively, 194<sup>11</sup> and 370<sup>12</sup> non-obese female European Caucasians. Here, we have investigated association of four *POMC* SNPs with anthropometric variables, body fat and serum leptin in 2758 normal weight Caucasian female twins (mean age  $47.4 \pm 12.5$  years), selected from the St Thomas' UK Adult Twin Registry (Twins UK) on the basis of available fasting plasma leptin data. In the *POMC* 5' flanking region, we tested *RsaI* and a second rare SNP, sited 630 bp upstream of the transcription start site in the 5'UTR, GenBank Accession V01510 nt 51 G/C (not on the NCBI database), found at a frequency of 1.9% in obese Caucasians.<sup>11</sup> In the 3'UTR, we tested 8246 C/T and another SNP 281 bp upstream, GenBank Accession V01510 nt 7965 C/T; dbSNP rs2071345, found at a frequency of 1.0% in obese Caucasians<sup>16</sup> but at 29.0% in lean Japanese<sup>17</sup> (named 7285 C/T on the early numbering system<sup>10</sup>). Our powerful study offered several prospects: (1) replication of the strong association of 8246C/T with serum leptin in normal Caucasian women; (2) exposure of small effects of the common SNPs that may have remained undetected previously; (3) exposure of strong

effects of rare alleles untested in a normal population and (4) a more rigorous test of the reported *RsaI/8246C/T* haplotype association with serum leptin in normal subjects.

## Materials and methods

### Subjects

The St Thomas' UK Adult Twin Registry (Twins UK) comprises unselected, mostly female volunteers ascertained from the general population through national media campaigns in the UK.<sup>18</sup> Means and ranges of quantitative phenotypes in Twins UK are normally distributed and similar to the age-matched general population in the UK.<sup>19</sup> The general characteristics of the subjects are given in Table 1. The majority of these twins also had measures of total and central body fat obtained by DXA body composition scans. Serum leptin, anthropometric and body fat variables were all strongly correlated, with correlation coefficients ranging from 0.68 to 0.94. Informed consent was obtained from participants before they entered the study and approved by the local research ethics committee.

### Zygosity, body composition and biochemical analyses

Zygosity was determined by standardised questionnaire and confirmed by DNA fingerprinting. Body composition was measured by DXA (Hologic QDR-2000, Vertec, Waltham, MA, USA).<sup>20</sup> Serum leptin concentration was determined after an overnight fast using a radioimmunoassay (Linco Research, St Louis, MO, USA).

### Genotyping

SNPs were genotyped using the PSQ96 HS 96A instrument (Pyrosequencing AB, Uppsala, Sweden).

**Table 1** General characteristics of subjects

Variable	Values
<i>n</i> <sup>a</sup>	2758
MZ/DZ subjects	846/1912
Age, years	$47.4 \pm 12.5$
Postmenopausal, %	47.8
Leptin, ng/ml	$16.5 \pm 12.0$
BMI, kg/m <sup>2</sup>	$24.8 \pm 4.4$
Weight, kg	$65.5 \pm 12.0$
Waist, cm	$78.3 \pm 10.4$
Total fat, kg	$23.3 \pm 8.9$
Total fat, %	$35.3 \pm 8.1$
Central fat, kg	$1.32 \pm 0.74$
Central fat, %	$30.6 \pm 11.5$

<sup>a</sup>Number of subjects with leptin data and genotype data on at least one SNP.

Mean  $\pm$  SD is shown, unless indicated otherwise.

**PCR** Template PCR and sequencing primers are given in Table 2. Primers were obtained from Sigma Genosys (Haverhill, UK). PCR reactions were set up in 96-well microplates (Thermo Life Sciences, Basingstoke, UK). Each 10 µl reaction contained 10 ng genomic DNA, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris pH 8.3, 0.01 % gelatin, 200 µM each dNTP, 4 pmol each primer and 0.2 U of *RedTaq* polymerase (Sigma-Aldrich, Poole, UK). Templates were amplified on an MJ Research DNA Engine Tetrad thermo-cycler (GRI, Braintree, UK). PCR cycling conditions were as follows: 95°C for 10 min, 95°C for 45 s, anneal temperature for 45 s, 72°C for 45 s for 50 cycles and 72°C for 10 min. Anneal temperature for the *RsaI* SNP PCR was 68°C and 56°C for the other SNP PCRs.

**Pyrosequencing** Biotinylated PCR product (10 µl) was immobilised onto sepharose streptavidin-coated beads (Amersham Bioscience, Amersham, UK) suspended in binding buffer (10 mM Tris-HCl, 2 M NaCl, 1 mM EDTA, 0.1% Tween 20, pH 7.6) and water to a total volume of 70 µl, at room temperature (21°C) for 5 min. Single-stranded (ss) DNA was obtained by washing the immobilised PCR product for 5 s successively in 70% ethanol, 0.2 M NaOH and washing buffer (10 mM Tris-acetate) followed by release into 12 µl annealing buffer (20 mM Tris-Acetate, 5 mM magnesium acetate, pH 7.6) containing 3.0 µM sequencing primer in a 96-well PSQ HS plate. The primer was annealed to the ssDNA template by heating the plate at 80°C for 2 min, then allowing it to cool to room temperature (21°C). Pyrosequencing was carried out using the PSQ96 HS 96A instrument and the SNP Reagent kit containing dNTPs, enzyme and substrate mixtures (Pyrosequencing AB, Uppsala, Sweden), according to manufacturer's protocols.

**Statistical analysis**

Regular association analyses were performed using Generalised Estimating Equations, a technique that provides valid inference on regression parameters in related individuals, that is, estimators and their standard errors are asymptotically consistent.<sup>21</sup> For individual SNP association analyses, codominant, dominant and recessive models were tested. Details of our approach to test the association of statistically inferred haplotypes with continuous traits have been described previously.<sup>22</sup> In short, we used haplotype trend regression as outlined by Zaykin *et al*,<sup>23</sup> with the probabilities of haplotype pairs estimated by PHASE 2.0 software.<sup>24,25</sup> We further investigated whether the effect of the *POMC* gene on leptin or body composition variables was dependent on age and menopausal status by testing interactions of individual SNPs and haplotypes of the *POMC* gene with these variables. To control for population stratification bias, DZ twin pairs discordant for genotype were also used in a sib-TDT association analysis as described elsewhere.<sup>26,27</sup> The program ARLEQUIN 1.1 was used to test deviation of the genotype

**Table 2** Pyrosequencing primers

dB SNP	GenBank	Alleles	Template PCR*	Template primers	Pyrosequencing primer
None	V01510 nt 51	G/C	<u>acagcatcacctctccccatttaattggttaggittaacagg/cacttttccctt</u> gagcttgggacagaaggaggcctcccctcaaacaggcccttggagag caggccaggaggagcagtgcaactcacttcacccaaccaagaagcgct cctgactctgctccccctccccccraaagtggaacagagagaaat	F: 5'-acagcatcacctctccc-3' R: 5'-biotin-attctctgttccactttg-3'	F: 5'-attaatggtttaggta-3'
rs2071345	V01510 nt 7965	C/T	<u>ttaagaggagctgactggccagcgaactcgggaggagatggccccgac</u> ggccctgc/tgatgacggccagggccaggccgacctggagcacagc tgctgtgctggccgagaagaagatcagggcccccatacagatggagcact	F: 5'-biotin-ttaagaggagctgactgg-3' R: 5'-aagtgctccatcctctgtaggg-3'	R: 5'-ccctgcgcccgtcat-3'
rs1042571	V01510 nt 8246	C/T	<u>aaacgcatacatcaagaacgctcaagaaggcggagtgaggccacagcg</u> gccccaggctaccctccccagagggtcagcccaagcccttctc/t cctgcccctgctgcgctccagccttgggggctgggagataatcagcct cittaagctgctgtagt	F: 5'-aaacgcatacatcaagaacg-3' R: 5'-biotin-aactacaggcagctttaaagg-3'	F: 5'-aggtcgaccaccaaaa-3'
rs3754860	NT 022184 nt 4209187 ( <i>RsaI</i> )	G/A	<u>gtgaaccctctctaccacaaacaaacaaacaaacaaacaaacaaac</u> aaaaccacaaatgctggcagtggtgcatgctatctggtttccag g/atactcaggaggctgagcatgaggtactcactgagcc	F: 5'-biotin-gtgaaccctctctaccacaaac-3' R: 5'-ggctcagtgatcctcatgct-3'	R: 5'-cttcagccctctgagta-3'

distribution from Hardy–Weinberg equilibrium. The test is analogous to Fisher’s exact test on a two-by-two contingency table but extended to a triangular contingency table of arbitrary size and is carried out using a modified version of the Markov-chain random walk algorithm.<sup>28</sup> To prevent inflated significance caused by the dependency between genotypes of twin pairs, this test was performed in a sample consisting of one twin of each MZ and DZ pair chosen at random as well as all singleton DZ twins.

allele frequencies, for a population in Hardy–Weinberg equilibrium.

### Association between POMC 8246 C/T genotype and phenotypes

The total number of subjects genotyped for the POMC 8246 C/T SNP was 1737, comprising dizygotic (DZ) twins in pairs, single representatives from each of the monozygotic (MZ) pairs and DZ singletons. The allele frequency was 18.1% (95% CI: 16.8–19.4%) (Table 3). A total of 2072 subjects were tested for association with quantitative variables, comprising the 1737 genotyped plus the second twin in each MZ pair. Variations in leptin and body composition phenotypes with respect to genotype in the twins are shown in Table 4. Three models were used to

### Results

The allele and genotype frequencies of the four SNPs studied are given in Table 3. Distribution of genotypes was not significantly different from that predicted, from these

**Table 3** Genotype and allele distributions

SNP	11	Genotype			Total	Minor allele frequency and 95% CI (%)	P-value for H–W test <sup>a</sup>
		12	22				
51 G/C	1728	36	0	1764	1.02 (0.72–1.41)	NS	
7965 C/T	1902	15	0	1917	0.39 (0.22–0.64)	NS	
8246 C/T	1153	531	48	1737	18.1 (16.8–19.4)	NS	
RsaI	954	817	161	1932	29.5 (28.0–30.9)	NS	

<sup>a</sup>Hardy–Weinberg (H–W) equilibrium tested in one of each MZ pair, one of each DZ pair and all singleton DZ twins.

**Table 4** Variation in leptin and body composition phenotypes with respect to POMC 8246 C/T genotype

Variable <sup>a</sup>	No./value	Full cohort Genotype			P <sup>b</sup> CC and CT vs TT	No. pairs	sib-TDT Genotype		P
		CC	CT	TT			CC and CT	TT	
Leptin, ng/ml	n Value	1383 16.7±12.6	635 16.4±11.0	54 19.0±13.5	0.059/0.095	27	14.1±8.8	21.4±14.7	<b>0.016</b>
BMI, kg/m <sup>2</sup>	n Value	1372 24.8±4.5	630 24.8±4.4	54 26.0±4.6	<b>0.021/0.032</b>	26	25.0±4.3	26.7±5.5	0.053
Weight, kg	n Value	1373 65.4±12.2	630 65.6±11.7	54 68.4±11.5	<b>0.045/0.060</b>	26	65.6±12.8	68.9±12.9	>0.10
Waist, cm	n Value	1344 78.5±10.5	618 78.0±9.9	53 80.2±10.1	<b>0.042/&gt;0.10</b>	27	77.4±10.8	81.8±11.1	<b>0.049</b>
Total fat, kg	n Value	1357 23.6±9.1	624 23.5±8.7	53 25.7±9.5	<b>0.017/0.046</b>	26	22.2±8.5	26.4±10.5	<b>0.037</b>
% Fat	n Value	1334 35.8±8.2	616 35.6±7.9	51 36.5±8.4	0.076/>0.10	25	34.2±7.3	37.2±9.0	>0.10
% Central fat	n Value	1344 31.4±11.7	624 31.3±11.4	52 32.1±12.4	>0.10/>0.10	25	28.4±11.8	31.7±12.6	>0.10
Central fat, kg	n Value	1344 13.5±7.3	624 13.2±7.2	52 14.4±8.7	>0.10/>0.10	25	11.9±7.5	14.3±8.7	>0.10

<sup>a</sup>Except % central fat and % total fat, all variables were log transformed to improve normality. Figures represent means±SD.

<sup>b</sup>Not adjusted/adjusted for age.

Significant P-values (<0.05) in bold.

compare mean phenotypic values with respect to genotype. Under the recessive model, there were a number of significant associations: BMI ( $P=0.017$ ), weight ( $P=0.043$ ), waist ( $P=0.042$ ) and total fat ( $P=0.017$ ), with a marginally significant association with serum leptin ( $P=0.059$ ). Weight, BMI and total fat associations remained (borderline) significant after adjustment for age (respectively,  $P=0.060$ ;  $P=0.032$  and  $P=0.046$ ). For all variables, TT homozygotes showed higher values. However, the proportions of total phenotypic variance explained were all less than 1%: leptin 0.06%, BMI 0.22%, weight 0.18%, waist 0.10% and total fat 0.15%. No interactions were found for age and menopause for any of the variables.

Population association tests are vulnerable to false positives created by population stratification and admixture. We therefore examined variation in mean phenotypes by sib-TDT in 25–27 pairs (dependent on phenotype data available), which were discordant for genotype (Table 4). Associations under the recessive model were maintained with BMI ( $P=0.053$ ), waist ( $P=0.049$ ) and total fat ( $P=0.037$ ) and a significant association was found with serum leptin ( $P=0.016$ ). The magnitude of the effects shown in the sib-TDT was, in general, higher than in the population association. For example, there was a difference of 4.2 kg fat between carriers and noncarriers of the C allele

in the sib-TDT compared to a difference of 1.9 kg between these groups in the population.

#### Association between POMC RsaI genotype and phenotypes

The total number of subjects genotyped for the POMC RsaI (+/-) SNP was 1932. The (-) allele frequency was 29.5% (95% CI: 28.0–30.9%) (Table 3).

A total of 2278 subjects were tested for association with quantitative variables. Variations in leptin and body composition with respect to genotype are shown in Table 5. Under the recessive model, there were significant associations with waist measurement ( $P=0.041$ ) and % central fat ( $P=0.015$ ). The latter remained significant after adjustment for age ( $P=0.036$ ) but disappeared in a sib-TDT based on 83 pairs (Table 5). The proportions of total phenotypic variance explained were less than 1%: waist 0.21% and central fat 0.15%. No interactions were found for age and menopause for any of the variables.

#### Test of association between POMC RsaI/8246 C/T haplotype and phenotypes

Pairwise linkage disequilibrium between the RsaI and 8246 C/T sites measured by  $D'$  was  $-0.78$  ( $P<0.0001$ ). The frequencies of the four POMC RsaI/8246 C/T haplotypes based on 1465 subjects (single representatives from each of

**Table 5** Variation in leptin and body composition phenotypes with respect to POMC RsaI genotype

Variable <sup>a</sup>	No./value	Full cohort			$P^b$ +/+ and +/- vs -/-	No. pairs	sib-TDT		P
		+/+	+/-	-/-			+/+ and +/-	-/-	
Leptin, ng/ml	n Value	1117 16.4±12.0	979 16.4±11.3	182 17.9±13.4	>0.10/>0.10	85	15.6±10.5	16.8±13.6	>0.10
BMI, kg/m <sup>2</sup>	n Value	1110 24.9±4.5	975 24.6±4.2	180 25.3±4.3	>0.10/>0.10	83	24.8±3.7	24.9±4.0	>0.10
Weight, kg	n Value	1111 65.5±12.1	975 65.3±11.7	180 65.3±10.8	>0.10/>0.10	83	64.5±9.2	64.8±10.5	>0.10
Waist, cm	n Value	1086 78.6±10.5	959 77.9±9.7	178 80.0±10.7	<b>0.041</b> />0.10	82	77.6±9.3	79.0±9.8	>0.10
Total fat, kg	n Value	1087 23.5±8.8	963 23.3±8.8	180 24.5±8.9	>0.10/>0.10	85	23.2±7.4	23.4±8.1	>0.10
% Fat	n Value	1070 35.7±8.0	945 35.3±8.0	178 37.1±8.4	0.084/>0.10	83	36.0±7.2	36.0±7.7	>0.10
% Central fat	n Value	1078 31.3±11.5	951 30.9±11.5	178 34.1±11.7	<b>0.015/0.036</b>	83	31.9±10.7	32.2±11.0	>0.10
Central fat, kg	n Value	1078 1.34±7.30	951 1.32±0.75	178 1.45±0.71	0.059/>0.10	83	1.34±0.70	1.36±0.65	>0.10

<sup>a</sup>Except % central fat and % total fat, all variables were log transformed to improve normality. Figures represent means ± SD.

<sup>b</sup>Not adjusted/adjusted for age.

Significant  $P$ -values ( $<0.05$ ) in bold.

**Table 6** POMC *RsaI*/8246 C/T haplotype frequencies

Haplotype	<i>RsaI</i>	8246 C/T	Frequency
1	+	C	0.539
2	+	T	0.174
3	-	C	0.275
4	-	T	0.012

Haplotypes determined by PHASE in 1465 subjects (one of MZ+singleton DZ+all DZT) with genotype data on both polymorphisms.

the monozygotic MZ pairs and all DZ) and determined by PHASE are shown in Table 6. No significant or borderline significant associations were found with any phenotypes with respect to haplotype (data not shown). Note that the *RsaI* (-) and 8246 T alleles that were found to be responsible for the effect on body fat-related variables in recessive models of the individual loci showed a trans-LD pattern ( $D' = -0.78$ ). That is, they do not usually occur together on the same chromosome (as can be clearly seen in Table 6), which makes it unlikely that the effect on body fat variables is due to LD between these two loci. To clarify whether the effects of these two polymorphisms were independent, we performed a multivariate regression analysis including the recessive effects of these two SNPs and observed that the significant results from the single SNP analyses of both 8246 C/T and *RsaI* remain unchanged.

#### Association between POMC 51 G/C and 7965 C/T genotypes and phenotypes

The total number of subjects genotyped for the 51 G/C SNP was 1764. The C allele frequency was 1.02% (95% CI: 0.72–1.41%) (Table 3). A total of 2129 subjects were tested for association with quantitative variables. The total number of subjects genotyped for the 7965 C/T SNP was 1917. The T allele frequency was 0.39% (95% CI: 0.22–0.64%) (Table 3). A total of 2275 subjects were tested for association with quantitative variables. There were no significant associations in the whole cohort and none in sib-TDT based on 17 pairs discordant for 51 G/C genotype or based on nine pairs discordant for 7965 C/T genotype.

#### Linkage disequilibrium between *RsaI*, 51 G/C and 7965 C/T and 8246 C/T loci

Pairwise LD was determined in 958 subjects (one MZ representative, DZ singletons and DZT in pairs) with genotype data on all four SNPs. Apart from the strong LD between *RsaI* and 8246 C/T noted previously, minimal LD was shown between other pairwise combinations (Table 7).

#### Discussion

Previous investigations of the twins have shown that total adiposity and central abdominal fat mass are under strong

**Table 7** Pairwise LD coefficients between the four SNPs<sup>a</sup>

	51 G/C	7965 C/T	8246 C/T	<i>RsaI</i> +/-
51 G/C		0.79	0.82	0.75
7965 C/T	-1.00		0.06	0.49
8246 C/T	-0.12	0.32		<0.0001
<i>RsaI</i> +/-	0.05	0.15	-0.78	

<sup>a</sup> $D'$  below diagonal and  $P$ -value above diagonal.

genetic influence.<sup>20</sup> Others, who have established that variation in leptin levels has a strong genetic component,<sup>7,9</sup> have cited POMC as a strong positional candidate for a major leptin QTL on chromosome 2. Two SNPs spanning the POMC gene, *RsaI* and 8246 C/T, have received most attention in tests of association with serum leptin levels; however, studies based on small samples (less than 350 subjects) have reported conflicting results. In nonobese Caucasian subjects, Echwald *et al*<sup>11</sup> found no association between 8246 C/T and body fat or fasting serum leptin in female subjects. In mixed sex subjects, Delplanque *et al*<sup>12</sup> found no association with BMI, but Suviolahti *et al*<sup>13</sup> discovered an association with serum leptin. We have found carriage of the 8246 T allele was significantly associated with higher mean BMI, weight, waist and total fat and marginally with higher leptin levels under a recessive model; however, the proportion of total phenotypic variance explained was less than 1% in all cases. BMI and total fat associations remained significant after adjustment for age. Although only 25–27 pairs were informative (ie, discordant for their genotype) for this test, the sib-TDT confirmed our significant associations with higher BMI, waist and total fat and revealed a significant association with leptin, and ruled out spurious associations caused by hidden population stratification. The magnitude of the effects shown in the sib-TDT was, in general, higher than in the overall sample, enabling us to find these significant effects in the relatively small TDT sample. This phenomenon, which we have observed before,<sup>22,26</sup> may be specific to using DZ twins for the sib-TDT. DZ twins may have added value as compared to ordinary sibs, because they are naturally matched for age and probably more closely matched for a range of environmental confounders. The within-DZ pair difference as used in the sib-TDT may, therefore, provide a more precise estimate of the genetic effect.

Our study has therefore exposed associations with BMI, serum leptin and total fat in normal individuals that were not detected in much smaller samples of normal female subjects studied by Echwald *et al*<sup>11</sup> and mixed subjects by Delplanque *et al*,<sup>12</sup> but has not replicated the strong association between the *RsaI* SNP and serum leptin found in obese mixed subjects by Hixson *et al*<sup>15</sup> or lean women by Suviolahti *et al*.<sup>13</sup> Analyses of association in mixed sex

samples is likely to be flawed, as sex-specific differences in leptin levels and patterns of fat deposition are well-known. The possible confounding effects of menopausal status may account in some part for variability between the findings in different female groups. The normal female subjects studied by Echwald *et al*<sup>11</sup> were aged 18–32 years and the average age of female subjects studied by Suviolahti *et al* was  $56 \pm 10.6$  years, representing a distinct difference in menopausal status. The average age of the Twins UK cohort was  $47.4 \pm 12.5$  years, with approximately equal numbers pre- and postmenopausal (Table 1). However, we tested interactions with age and menopausal status and found no effect.

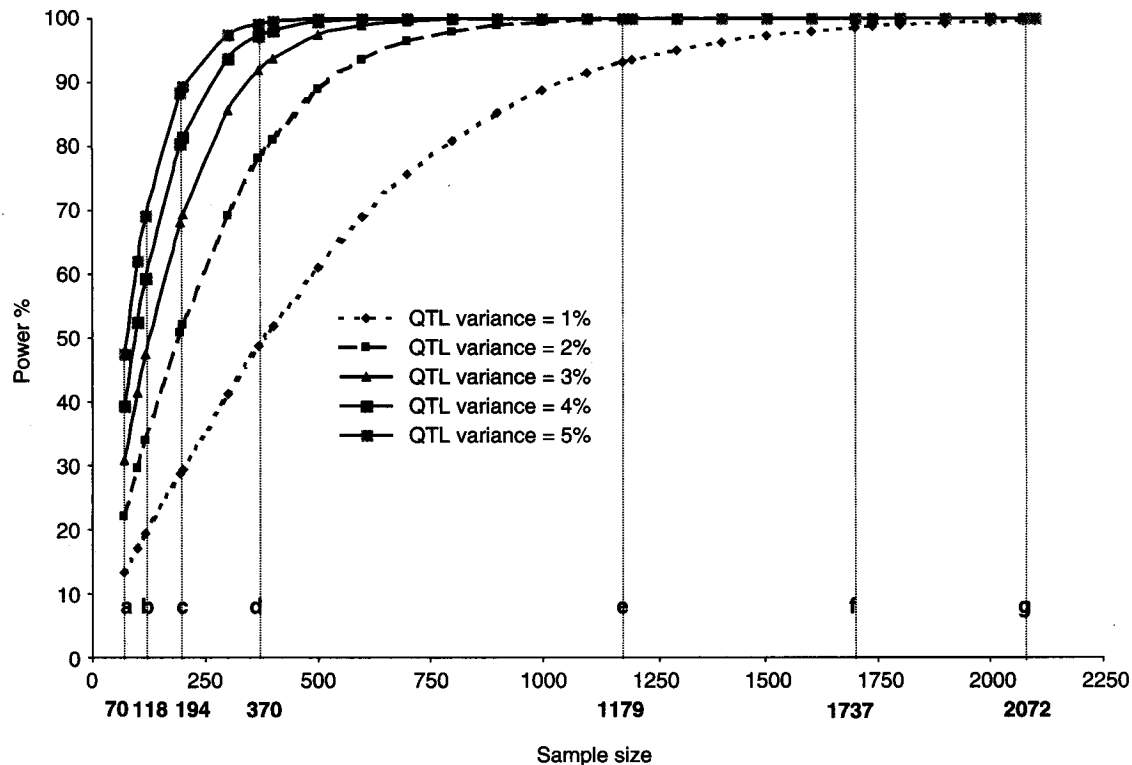
The only positive association found with the 8246 C/T SNP, by Suviolahti *et al*<sup>13</sup> in an even smaller mixed sample ( $P=0.003$ ,  $n=118$ ), proved to be stronger in the female subjects ( $P=0.004$ ,  $n=70$ ), but unlike us, they found that the C rather than the T allele was associated with higher serum leptin. However, the association with serum leptin in the twins was not as strong as that found by Suviolahti *et al*<sup>13</sup> in only 70 women. We found that the *RsaI* SNP (–) allele was significantly associated with higher waist measurement and % central fat, although the proportion of total phenotypic variance explained was less than 1%. The latter remained significant after adjustment for age but was not confirmed in the sib-TDT. Trends toward higher serum leptin, BMI, waist measurement and proportion of body fat associated with the (–) allele were apparent. Here, we are in agreement with Suviolahti *et al*,<sup>13</sup> who found that carriers of *RsaI* (–/–) had higher serum leptin levels than (+/–). In their study, in combination with 8246 CC genotype, the difference was highly significant in female subjects.

We found that linkage disequilibrium between the *RsaI* and 8246C/T loci was moderately strong. Rather than comparing genotype combinations, we determined haplotype frequencies in the twins using the PHASE 2.0 program, which takes account of indeterminate phase of alleles in heterozygotes, enabling full utilisation of population data to test association. In subjects with genotypes for both SNPs, we found no significant association between any of the four *RsaI*/8246 C/T haplotypes and any phenotypic parameter. However, multivariate regression analysis including the recessive effects of these two SNPs confirmed that the significant results from the single SNP analyses of both 8246 C/T and *RsaI* remain unchanged.

Suviolahti *et al*<sup>13</sup> failed to find any association between 8246 C/T or the 8246 C/T/*RsaI* genotype combination and serum leptin in obese subjects. However, Hixson *et al*<sup>15</sup> found in mainly obese Mexican Americans that the haplotype combinations of *RsaI* and 8246 C/T were significantly associated with serum leptin levels ( $P=0.001$ ,  $n=337$ ), with homozygotes of the *RsaI* (–)/8246 T haplotype having leptin levels almost two-fold

higher than homozygotes of the *RsaI* (+)/8246C haplotype. Suviolahti *et al*<sup>13</sup> on the other hand examined the effect of all *genotype combinations* on serum leptin levels. The genotype combination associated with the highest leptin level in lean subjects by Suviolahti *et al*<sup>13</sup> (*RsaI* (–/–) or (+/–)/C8246C) was not represented by the haplotype combination linked to highest leptin levels seen in the obese subjects by Hixson *et al*<sup>15</sup> (*RsaI*(–)/T8246T). This may, as was suggested,<sup>13</sup> relate to a fundamental difference in the obese *versus* nonobese condition, or differences in allele frequency or LD between the populations, but the relatively low sample size and the fact that actual haplotypes were not determined infers that association of an *RsaI*/8246C/T haplotype with serum leptin remains questionable in normal (lean) subjects. As our individual haplotype analyses did not reveal any strengthening of associations observed with either of the single SNPs 8246 C/T and *RsaI*, the haplotype does not appear to be in stronger LD with any causal SNP(s) than either 8246 C/T or *RsaI* alone. We therefore find ourselves at odds with the outcome of the previous study of the 8246 C/T SNP in lean Caucasian women<sup>13</sup> and our analyses of individual haplotypes does not support their reported association of serum leptin with *RsaI*/8246 C/T genotype combinations. Single haplotype frequencies calculated from the combination data given by Hixson *et al*<sup>15</sup> in Mexican Americans reveal only minor differences to our own, but may be relevant to the emergence of a haplotype association with serum leptin in these obese subjects.

However, issues of power may contribute more to variability in results than the definition of haplotype structure. Figure 1 shows the power of the current study, compared to that of previous studies, to detect QTL explaining 1–5% of the variance ( $\alpha=0.05$ ). Based on our sample size for the 8246 C/T SNP, the power of our study of twins will lie somewhere between the number of completely independent individuals (e:  $n=1179$ ) and our total number of twins (g:  $n=2072$ ). It is immediately obvious from Figure 1 that some previous studies were severely underpowered. Inconsistencies between our own and previous studies (and between previous studies) may, therefore, be due to false positives exacerbated by studies of small sample size, differences in LD between a causative mutation related to population histories or by hidden stratification within study populations. We have eliminated the influence of stratification and admixture in confirming associations of body fat measures with both SNPs, by use of the sib-TDT. However, we believe that anomalous associations may have arisen in unrepresentative small study samples, which we have not been able to replicate in our much larger cohort. Our study reiterates the need for adequate power in establishing SNP associations with quantitative phenotypes and the need to check spurious relationships that may owe their origins to a nonhomogeneous population.



**Figure 1** Power for association tests at different sample sizes to detect quantitative trait loci (QTL) explaining 1–5% of the variance ( $\alpha=0.05$ ). A codominant model with additive effects only (ie, dominance variance=0) is assumed. (a) Based on female subjects ( $n=70$ ) in Suviolahti *et al.*<sup>13</sup> (b) Based on total sample ( $n=118$ ) in Suviolahti *et al.*<sup>13</sup> (c) Based on total sample ( $n=194$ ) in Echwald *et al.*<sup>11</sup> (d) Based on total sample ( $n=370$ ) in Delplanque *et al.*<sup>12</sup> Based on sample size for the 8246 C/T polymorphism in the current study: (e) including one of each MZ pair, one of each DZ pair and all DZ singletons ( $n=1179$ ); (f) including one of each MZ pair, all DZ twins and all DZ singletons ( $n=1737$ ); (g) including all subjects ( $n=2072$ ).

We found that the other two SNPs, 51 G/C and 7965 C/T, previously reported at low frequency in obese subjects,<sup>11,16</sup> occurred at even lower frequency in our normal female subjects: 51 G/C at 1.0% and 7965 C/T at 0.4%. The inclusion of these two rare SNPs was aimed at exposing any relatively strong effect in potential regulatory regions (51 G/C in the promoter and 7965 C/T in the 3'UTR) by examining a large sample. There were no significant associations, although trends towards lower measurements of body fat were shown in carriers of the 51 C allele and higher levels in carriers of 7965 T. Apart from 8246 C/T and *RsaI*, we found absence of pairwise LD among the four *POMC* SNPs that we tested and the two rare SNPs are unlikely to mark significant etiological sites.

In conclusion, we have selected four polymorphisms spanning the *POMC* gene to test for associations with level of serum leptin and variables related to body weight and composition in over 2000 normal Caucasian female subjects. We have observed a number of significant associations of two common SNPs in untranslated regions, 8246 C/T and *RsaI*, with leptin and/or body fat deposition. No interactions were found for age and menopause for any of the fat variables, so observed effects

of the gene do not depend on age or menopausal state. Neither SNP has been shown by others to influence expression of *POMC* or the activity of the cleavage products, but could be in LD with a functional site. It has been suggested that any causative mutations are more likely to occur in regulatory sequences, because coding changes could affect more than one of the overlapping cleavage products and have far-reaching effects on other physiological processes.<sup>11</sup> The associations of BMI, weight and total fat with SNPs in regions flanking the *POMC* gene exposed in this powerful study suggest that control of *POMC* expression may be influential in the determination of body weight.

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