ORIGINAL ARTICLE

A rare variant of the leptin gene has large effects on blood pressure and carotid intima-medial thickness: a study of 1428 individuals in 248 families

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Background: Rare mutations in the leptin (*LEP*) gene cause severe obesity. Common polymorphisms of *LEP* have been associated with obesity, but their association with cardiovascular disease has been little studied. We have examined the impact of both common and rare polymorphisms of the *LEP* gene on blood pressure (BP), subclinical atherosclerosis as measured by carotid intima-medial thickness (CIMT), and body mass index (BMI) in a large family study.
Methods: Five polymorphisms spanning *LEP* were typed in 1428 individuals from 248 nuclear families. BP, CIMT, BMI, and plasma leptin were measured.
Results: The polymorphisms typed captured all common haplotypes present at *LEP*. There was strong association between a rare polymorphism in the 3′ untranslated region of *LEP* (C538T) and both pulse pressure ($p = 0.0001$) and CIMT ($p = 0.008$). C/T heterozygotes had a 22% lower pulse pressure and a 17% lower CIMT than C/C homozygotes. The polymorphism accounted for 3–5% of the population variation in pulse pressure and CIMT. There was no association between any *LEP* polymorphism and either BMI or plasma leptin level.
Conclusions: This large family study shows that the rare T allele at the C538T polymorphism of *LEP* substantially influences pulse pressure and CIMT, but does not appear to exert this effect through actions on plasma leptin level or BMI. This suggests that autocrine or paracrine effects in vascular tissue may be important physiological functions of leptin. This study also provides evidence that rare polymorphisms of particular genes may have substantial effects within the normal range of certain quantitative traits.

Leptin is the central hormone in the adiposity-sensing pathway. Circulating leptin is produced principally by adipocytes and functions as a satiety signal; coding or splice site mutations in the gene encoding leptin (*LEP*) which render the leptin protein unable to signal through its receptor, produce severe childhood onset obesity.[1 2] However, such coding sequence variation in *LEP* is extremely rare in general populations.[3 4] Evidence for association between commoner polymorphisms, mainly in the 5′ region of *LEP*, and obesity has been sought in several previous studies; significant association has been found in some but not in other studies,[5–8] and this issue remains controversial.

Although obesity is a risk factor for a variety of conditions, cardiovascular disease is the principal source of morbidity and mortality in obese persons. Thus far, the cardiovascular effects of leptin, and the possible existence of obesity independent associations between polymorphisms of *LEP* and cardiovascular risk, have been relatively little studied. Blood pressure (BP) is a major risk factor for cardiovascular disease, and the contribution of genes to the population variability of BP is significant.[9] A number of cross sectional studies suggest that increased arterial stiffness, measured by a variety of techniques, is an independent risk factor for cardiovascular disease[10–14]; pulse pressure is a simply measured indicator of increased arterial stiffness with a prospectively validated relationship to cardiovascular risk in large numbers of subjects.[15] Measurement of the intima-medial thickness (IMT) of the carotid artery is a non-invasive method of assessing the extent of atherosclerosis in pre-symptomatic subjects which is strongly associated with the future occurrence of cardiovascular events in prospective studies.[16] We have examined the relationship between both common and rare polymorphisms of the leptin gene and BP, carotid intima-medial thickness (CIMT), and body mass index (BMI) in a large family based association study.

METHODS

The collection strategy of this family study has been previously described.[17 18] Briefly, families were ascertained between 1993 and 1997 through a proband with essential hypertension. In order to be suitable for the study, families were required to consist of at least three siblings clinically assessable for BP if at least one parent of the sibship was available to give blood for DNA analysis, and to consist of at least four assessable siblings if no parent was available for DNA analysis. Families were extended to include the nuclear families of additional individuals in the sibship who were hypertensive, where applicable. Thus, the majority (65%) of the individuals in the family collection have BP within the normal range, and most families consist of single sibships.

Blood pressure was measured using ambulatory monitoring for a period of 24 h (A&D TM2421, Takeda Medical, Toyko, Japan) as previously described.[19] A full clinical history was taken, which included the subject's medical history and lifestyle factors including consumption of alcohol and tobacco, and habitual physical exercise. Anthropometric

Abbreviations: BMI, body mass index; BP, blood pressure; CIMT, carotid intima-medial thickness; IMT, intima-medial thickness; WHR, waist to hip ratio

Table 1 Primers and restriction enzymes used for genotyping

Polymorphism	Forward primer	Reverse primer	Restriction enzyme
G2548A	5′ TTTCCTGTAATTTTCCCGTGAG3′	5′AAAGCAAAGACAGGCATAAAAA3′	*Hha*I
C188A	5′CAACGAGGGCGCAGCCGTAT3′	5′AGTGTGCACCTCGCGGGGCCT3′	*Asc*I
A19G	5′GCCCCGCGAGGTGCACACTG3′	5′GGGCCCTGTGGCCTGCCAAG3′	*Msp*A1I
rs2060713	5′CCAGGCCTTGATTAAAGGAG3′	5′CATTTAGGAGCTGCCATTTTC3′	*Bsi*HKAI
C538T	5′CGACCTGGAGAACCTCCG3′	5′GTCCTGGATAAGGGGTGT3′	*Hpy*CH4IV

measurements including height, weight, and waist and hip measurement were noted (waist measured at the natural waist, and hip measured at the level of the greater trochanters). Blood was drawn into a variety of anticoagulants for plasma and DNA analysis. In all, 1428 individuals from 248 families participated in the study. Between 1997 and 2000, 955 individuals from 220 families re-attended for ultrasonic measurement of CIMT. This was performed using a 7.5 MHz linear array transducer on a Hewlett-Packard Sonos 5500 machine, as previously described.[20–22] A total of 856 individuals from 220 families had technically satisfactory measurements.

Plasma leptin was measured on heparinised samples using a commercially available ELISA kit (Linco Research, St Charles, MO) with sensitivity 0.5 ng/ml, intra-assay precision 2.6–4.6%, and inter-assay precision 2.6–6.2%. Five polymorphisms spanning the leptin gene in non-translated sequence were genotyped, four of which (G2548A, C188A, A19G, C538T) had been studied by previous investigators, and one of which (rs2060713, a C/T substitution in intron 1) had not previously been studied (fig 1). All polymorphisms were typed by PCR followed by restriction enzyme digestion and agarose gel electrophoresis to resolve fragments, using primer pairs and restriction enzymes shown in table 1. Controls of known genotype were included in each genotyping plate.

Mendelian inheritance of all genotypes was checked using PedCheck.[23] Possible genotyping errors not producing Mendelian inconsistencies were detected by examining the families for recombination in this short genomic segment using Merlin.[24] Haplotype frequencies across the leptin gene were calculated using Fugue.[25] Phenotypes of interest were examined for normality and log transformed where necessary. Significant covariates of these phenotypes were then determined by linear regression using MINITAB; the adjusted values from these regressions were used in the genetic analysis. Association between genotypes at the typed markers and the adjusted phenotypes was assessed in the families by calculating identity by descent vectors for each individual using Merlin followed by variance components analysis using QTDT.[26] In the present analyses, only those individuals in whom an ambulatory BP recording without drug treatment for hypertension was available were included. Since previous reports had suggested association of *LEP* polymorphisms with BMI either only in men or only in women, subsidiary analyses involving men and women separately were performed for BMI. Bivariate models to assess the evidence for pleiotropic effects of leptin variants were fitted using PAP version 5.0.[27]

RESULTS

Baseline demographics of the population are shown in table 2. The median BMI was representative of a UK general population unselected for obesity, and the median IMT values were within the normal population range (0.4–1.0 mm). Age, gender, alcohol consumption (in units per week), smoking (coded current/former/never), and habitual physical exercise accounted for 5–50% of the population variability in the different phenotypes of interest (table 2).

Genotyping was successful in >97% of family members at all polymorphisms, with insufficient remaining DNA sample the principal reason for dropout. The estimated genotype error rate was <1% for all polymorphisms. Allele frequencies at all polymorphisms satisfied Hardy-Weinberg equilibrium, and agreed closely with allele frequencies described at these polymorphisms in Caucasians in previous studies (table 3). The T allele of the C538T polymorphism in the 3′UTR of the *LEP* gene (*LEP*C538T) was rare, occurring in only 14 heterozygote members of the population from seven families and no homozygotes ($p_T = 0.005$). Linkage disequilibrium in the region was strong; only five of a possible 32 haplotypes had frequencies >0.6% (table 4). These haplotype data are in close agreement with a recent report from Jiang *et al* in a smaller but more intensively genotyped Caucasian population.[8]

No highly significant association between any of the polymorphisms and either systolic or diastolic BP was observed, though there was a borderline trend towards lower

Table 2 Characteristics of the study population

Variable	n	Min	LQ	Median	UQ	Max	R^2†
Age (years)	1425*	18.7	35.7	50.9	60.9	90.7	–
BMI (kg/m^2)	1402	16.7	23.1	25.4	28.2	51.8	15.2
WHR	1357	0.56	0.78	0.85	0.91	1.22	48.7
Plasma leptin (ng/μl)	1319	1.1	4.6	8.6	15.3	116.6	46.4
Daytime systolic (mm Hg)	958	94.2	121.1	131	144.1	214.0	20.4
Daytime diastolic (mm Hg)	958	54.0	72.0	78.6	88.0	119.9	17.9
Daytime pulse pressure (mm Hg)	958	29.0	45.0	51.4	58.4	105.0	14.2
Clinic pulse pressure (mm Hg)	928	24.0	43.7	50.5	60.9	112.0	21.8
Nighttime pulse pressure (mm Hg)	770	9.0	40.0	46.0	52.7	86.0	4.5
Mean IMT (mm)	854	0.42	0.65	0.76	0.91	2.17	38.9
Max IMT (mm)	856	0.44	0.71	0.83	1.00	2.53	37.3

*Of which 52.4% were female and 36.1% were classified as hypertensive; †proportion of variability explained by correction for environmental variables (that is, age, gender, alcohol consumption, smoking behaviour, exercise taken). All variables were log transformed before correction except WHR. This was done to approximately normalise the distributions.
LQ, quartile; UQ, upper quartile; WHR, waist to hip ratio.

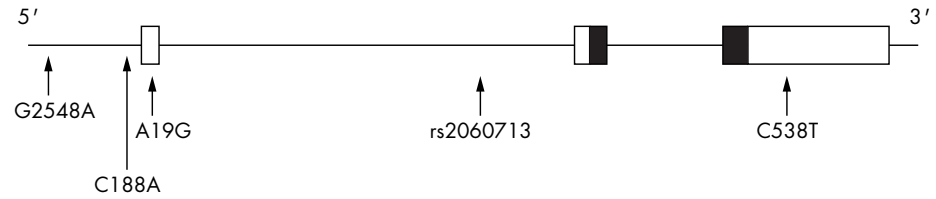

Figure 1 Position of five polymorphisms typed across 20 kb spanning the leptin (*LEP*) gene on chromosome 7. Black shading indicates coding sequence.

systolic BP with the T allele of *LEP*C538T ($p = 0.025$). The T allele of *LEP*C538T was highly significantly associated with a lower pulse pressure ($p = 0.0001$): even though Bonferroni correction for multiple analyses is almost certainly over-conservative in this situation,[28] this p value remains significant after such correction for 15 analyses (corrected p value = 0.005). CT heterozygotes had a 22.2% (standard error 4.8%) lower pulse pressure than CC homozygotes. The T allele was also associated with a significantly lower CIMT ($p = 0.0076$); CT heterozygotes had a 17.6% (6.2%) lower maximum CIMT. CIMT and pulse pressure were modestly, though significantly, correlated ($r = 0.15$, $p<0.0001$). We examined the evidence for pleiotropic effects of *LEP*C538T on these two traits using PAP. In a bivariate, measured genotype model of daytime pulse pressure and CIMT, the C538T variant was significantly associated with both traits ($p<0.05$); a minor proportion of the correlation between the traits was accounted for by the pleiotropic effects of the variant (table 5). *LEP*C538T accounted for 3–5% of the observed population variation in both pulse pressure and carotid IMT. There was no significant association between any *LEP* polymorphism and BMI either in the whole population, or in men and women considered separately. Nor was there significant association between any *LEP* polymorphism and plasma leptin level.

DISCUSSION

This large family based association study shows that the rare T allele of the C538T polymorphism in the 3′UTR of the *LEP* gene is associated with lower arterial stiffness (as measured by pulse pressure) and lower carotid IMT. Although this variant accounted for only about 5% of the population variability of these traits (because of its low frequency), it had a large effect in carriers, lowering both pulse pressure and IMT by around 20%. Such effects would be expected to have clinical significance in carriers with respect to risk of cardiovascular events.[15 16] Although *LEP*C538T is non-coding, similarly positioned variants in the 3′ UTR of other genes are known to have important effects on their expression.[29 30] *LEP*C538T was not associated with BMI or with plasma leptin level, suggesting that its effects on arterial stiffness and atherosclerosis may be mediated through a local (autocrine or paracrine) mechanism in vascular tissue. We found evidence in favour of a pleiotropic effect of *LEP*C538T on both pulse pressure and carotid IMT, suggesting that leptin may be involved in a pathway common to the development of increased arterial stiffness and the development of atherosclerosis. This is supported by evidence that leptin activates platelets and promotes thrombosis and neointimal thickening after vascular injury in mouse models, and thus may be involved in atheromatous plaque growth by repeated rupture and repair.[31 32] In the only previous report involving *LEP*C538T, no association between genotype and obesity was found in 200 obese and 65 non-obese Finnish subjects.[33] However, that study did not examine cardiovascular risk phenotypes. Only one previous, much smaller study has investigated the relationship between leptin polymorphisms and BP. Shintani *et al* investigated a tetranucleotide repeat in the 3′ region of *LEP* in a study of 205 hypertensive and 117 normotensive subjects; when alleles at this repeat were categorised into shorter and longer groups, a modest association between genotype and hypertension was demonstrated.[34] No previous study has examined the relationship between *LEP* polymorphisms and the severity of atherosclerosis as measured by carotid IMT.

The polymorphisms we typed at *LEP* captured all common haplotype variation across the gene, and no association between genotype at any polymorphism and either BMI or plasma leptin was present. Several previous studies have examined the relationship between leptin polymorphisms and obesity phenotypes, and the results are discordant, with some studies claiming association and others not. With the exception of the recent study by Jiang *et al*,[8] the present study is the only one to have assessed all common haplotypes of *LEP*, and it is also the largest study so far. It should, however, be noted that most previous studies have selected obese cases

Table 3 Genotype and allele frequencies at five SNPs of the *LEP* gene

SNP	Genotype	Frequency	
G2548A	G/G	406	$p_G = 0.546$
	G/A	704	$p_A = 0.454$
	A/A	279	
	All	1389	
C188A	C/C	1243	$p_C = 0.947$
	C/A	148	$p_A = 0.053$
	A/A	0	
	All	1391	
A19G	G/G	548	$p_G = 0.619$
	G/A	630	$p_A = 0.381$
	A/A	217	
	All	1395	
2060713	C/C	1241	$p_C = 0.951$
	C/T	129	$p_T = 0.049$
	T/T	3	
	All	1373	
C538T	C/C	1362	$p_C = 0.995$
	C/T	14	$p_T = 0.005$
	T/T	0	
	All	1376	

Table 4 Common haplotypes spanning the *LEP* gene

Haplotype frequency	G2548A	C188A	A19G	rs2060713	C538T
0.40	A	C	G	C	C
0.31	G	C	A	C	C
0.16	G	C	G	C	C
0.05	A	C	A	C	C
0.04	G	A	G	T	C

Table 5 Bivariate measured genotype analysis of *LEP*C538T polymorphism, pulse pressure, and carotid IMT

Trait		Estimate (SE)	95% CI
Day pulse pressure	Displacement*	1.00 (0.31)	0.39 to 1.61
	Polygenic h2†	0.24 (0.06)	0.12 to 0.36
Mean IMT	Displacement	0.90 (0.36)	0.19 to 1.61
	Polygenic h2	0.20 (0.07)	0.06 to 0.34
	Residual correlation	0.13 (0.04)	0.04 to 0.21

*Displacement is expressed in standard deviations between genotype means; †polygenic h2, residual polygenic heritability after accounting for effect of *LEP*C538T.
SE, standard error.

and compared them with non-obese controls; even among studies of "normal" individuals, the average BMI has tended to be higher than in our population (for example, a mean BMI of 29.5 in the study of Jiang *et al*, compared with 25.4 in the present study). Thus, our findings do not rule out variation in *LEP* as a cause of obesity, but suggest that such variation does not contribute significantly to the determination of BMI in those classified normal or overweight (BMI 19–29), some 75% of the UK population.[35]

Our findings may have more general implications. If a substantial proportion of the genetic influence on quantitative traits derives from multiple large displacement rare variants (such as *LEP*C538T), approaches focused on common variants alone may not be optimal. Though the impact of rare alleles on most quantitative traits has not been systematically evaluated, rare alleles with substantial phenotypic effects were recently shown to contribute significantly to low plasma HDL-C levels in the general population.[36] At least with respect to BP and atherosclerosis phenotypes, the present data suggest that full evaluation of both common and rare variants in candidate gene studies may be prudent in order that relatively large effects are not overlooked. With respect to the *LEP* gene, resequencing in cohorts selected to be extreme for the phenotypes we have studied, and screening of much larger populations (tens of thousands) to confirm the effect of the T allele in TT homozygotes would be important future experiments. Further in vitro and animal model research will also be necessary to discover how the T allele is involved in the regulation of BP and atherogenesis susceptibility.

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Ethics: The investigation corresponds to the principles outlined in the Declaration of Helsinki, and was approved by the appropriate local research ethics committees in Newcastle upon Tyne and Oxford, UK.

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ECHO

The spectrum of *Notch3* mutations in 28 Italian CADASIL families

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Please visit the *Journal of Medical Genetics* website [www.jmedgenet.com] for a link to the full text of this article.

Background: Cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy (CADASIL) is a cause of hereditary cerebrovascular disease. It results from mutations in the *Notch3* gene, a large gene with 33 exons. A cluster of mutations around exons 3 and 4 was originally reported and limited scanning of these exons was suggested for the diagnosis in most cases.

Objective: To report *Notch3* mutation analysis in 28 unrelated Italian CADASIL families from central and south Italy.

Results: The highest rate of mutations was found in exon 11 (21%) and only 18% of mutations were in exon 4. This may be related to the peculiar distribution of *Notch3* mutations in the regions of origin of the families.

Conclusions: The results suggest that limited scanning of exons 3 and 4 is inadvisable in CADASIL cases of Italian origin.

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