Journal of Human Genetics (2014) 59, 623–629

www.nature.com/jhg

ORIGINAL ARTICLE

# Novel locus for fibrinogen in 3′ region of *LEPR* gene in island population of Vis (Croatia)

Željka Tomas, Matea Zajc Petranović, Tatjana Škarić-Jurić, Ana Barešić, Marijana Peričić Salihović and Nina Smolej Narančić

**Leptin, a possible mediator between energy homeostasis, inflammation and cardiovascular disease (CVD), acts via leptin receptors. We investigated association of single-nucleotide polymorphisms (SNPs) and haplotypes of the leptin receptor gene (*LEPR*) with several CVD risk factors: body mass index, waist circumference (WC), serum lipids, fibrinogen and C-reactive protein levels. Thirty-one SNPs in and near *LEPR* gene were analyzed in 986 inhabitants of the island of Vis, Croatia and 29 SNPs in the inland sample ($N = 499$). We assessed linkage disequilibrium (LD), SNP and haplotype associations with the selected phenotypes. rs4291477 significantly associated with fibrinogen ($P = 0.003$) and rs7539471 marginally significantly with high-density lipoprotein ($P = 0.004$), but only in the Vis sample, while rs10493384 marginally significantly associated with triglyceride levels ($P = 0.006$) in the inland sample. SNPs were grouped into eight LD blocks in Vis and in seven blocks in the inland population. Haplotype A-C-A-A-G-A in block 5 in Vis (rs1782754, rs1171269, rs1022981, rs6673324, rs3790426, rs10493380) and haplotype A-A-A-A in block 4 in the inland data (rs1782754, rs1022981, rs6673324, rs1137100) were nominally associated with WC, $P = 7.085 \times 10^{-22}$ (adjusted $P = 0.0979$) and $P = 5.496 \times 10^{-144}$ (adjusted $P = 0.1062$), respectively.**
*Journal of Human Genetics* (2014) **59,** 623–629; doi:10.1038/jhg.2014.82; published online 9 October 2014

## INTRODUCTION

Cardiovascular diseases (CVDs) are the leading cause of morbidity and mortality in the world.[1] The majority of CVDs is caused by risk factors such as obesity, hypertension, diabetes, dyslipidemia and low-grade systemic inflammation. Furthermore, these risk factors have synergistic influence, conferring cardiovascular risk beyond that which is associated with the sum of their individual effects.[2]

Leptin is an adipocyte-derived protein with an important role in the regulation of food intake, metabolism, reproductive and immune functions. It is considered to be a plausible candidate for a mediator between the energy homeostasis, the inflammation and the CVD. Leptin exerts its biological function through the activation of leptin receptors (LepRs). LepR, encoded by a single gene on the chromosome 1p31 (leptin receptor gene (*LEPR*)), is a single-transmembrane protein with several alternatively spliced isoforms. All LepR isoforms share the same extracellular leptin-binding domain but differ in the length of the cytoplasmatic signal-transducing domain.[3,4] Short isoform is involved in the transport of leptin across the blood–brain barrier, whereas long isoform, abundantly expressed in the hypothalamus, modulates the expression of neuropeptides controlling satiety and hunger.[5]

Increased circulating leptin levels, commonly found in obesity as a reflection of the increased production in adipose tissue, indicate a failure to signal satiety and curtail the progression of obesity. In addition, clinical trials in hyperleptinemic obese patients have yielded variable, at best modest, results, giving rise to the leptin resistance hypothesis.[6,7] Both the reduced transport across blood–brain barrier and the impaired signalling are proposed possible causes of leptin insensitivity in obese subjects with increased blood leptin levels.[8] Beside its role in the obesity, leptin is a key factor in stimulating inflammatory response.[9,10] Furthermore, the results of several animal model studies imply that leptin has prothrombotic effects.[11,12] In conclusion, numerous evidences strongly imply that leptin has important role in CVDs, and, indeed, the elevated serum leptin level was found to be independently associated with CVDs in several studies.[13–20]

However, regardless of listed strong indications of the involvement of leptin–leptin receptor system in the etiology of CVD, the results of studies aiming to elucidate association of leptin and *LEPR* gene single-nucleotide polymorphisms (SNPs) with CVDs and CVD phenotypes are inconclusive and ambiguous.[21,22] The most of the association studies are designed on the basis of one-polymorphism-at-a-time approach, ignoring the fact that SNPs are not independent, but that the variation in some population is structured into haplotype blocks inherited as a unit. In addition, testing haplotypes instead of SNPs reduces dimensionality of association tests by reducing the total number of statistical tests, as well as reducing the number of possible combinations of genotypes when testing combined effect of two or more SNPs, which may contribute to increased statistical power.[23] In this study, we investigated both individual association of SNPs and

Institute for Anthropological Research, Gajeva 32, Zagreb, Croatia
Correspondence: Dr Ž Tomas, Institute for Anthropological Research, P.O. BOX 290, Ljudevita Gaja 32, 10 000 Zagreb, Croatia.
E-mail: ztomas@inantro.hr
Received 19 March 2014; revised 18 August 2014; accepted 2 September 2014; published online 9 October 2014

association of haplotypes in *LEPR* region with CVD risk phenotypes in an isolated population of island of Vis, Croatia, characterized by a very high prevalence of obesity, hypertension and metabolic syndrome.[24,25]

## MATERIALS AND METHODS

For this study, we used genotypic and phenotypic data of 986 participants randomly selected from the island of Vis (Croatia) adult population.[26] Sample consisted of 400 males and 586 females aged 18–93 years (mean age 56.29 years), and all the participants gave informed consent. The study was approved by the Ethical Committee of the Medical School, University of Zagreb and the Multi-Centre Research Ethics Committee for Scotland.

The analyses were also conducted in the inland sample from Split, Croatia (The CROATIA-Split study). Split is the closest mainland large town (population over 178 000) that has a similar climate and ecological environment as Vis, with the only exception of not being isolated geographically. The sample includes randomly selected participants whose ancestry does not trace back to either Vis or any other island. It consisted of 286 females and 213 males; aged 18–85 years (mean age 49.04 years). The CROATIA-Split study was approved by the appropriate ethics committees: the Ethics Committee of the University of Split Medical School and the South East Scotland Research Ethics Committee.

In both the Vis and Split population, blood samples were obtained by venipuncture in fasting individuals (20 ml EDTA (BD Vacutainer Systems, Franklin Lakes, NJ, USA) for DNA extraction, 4.5 ml citrate for clotting factors and 10 ml clotted blood for serum biochemistry). Biochemical analyses included determining cholesterol (total, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol), triglyceride, fibrinogen and C-reactive protein (CRP) levels. Serum for biochemical analyses of HDL, LDL, total cholesterol and triglycerides was rapidly frozen, stored in 200 µl aliquots at − 20 °C and transported to a biochemical laboratory in Zagreb, Croatia. For the Vis sample, plasma used for the biochemical analyses of fibrinogen and CRP levels was stored in a freezer at − 80 °C and frozen transported to the Institute of Cardiovascular and Medical Sciences, Glasgow, Scotland. Fibrinogen was measured by the Clauss method using an MDA 180 coagulometer (Biomerieux, Marcy l'Etoile, France) with reagents from the manufacturer. The calibrant used was the 8th British Standard (NIBSC). Highly sensitive CRP was measured immunologically using the BNProSpec nephelometer (Dade Behring, Newark, DE, USA) using calibrants and reagents provided by the manufacturer. DNA was extracted from blood samples using Nucleon DNA purification kits (Tepnel, Manchester, UK). Genotyping was performed using the Illumina's Sentrix HumanHap300 Genotyping BeadChip (v1) comprising 317 503 SNPs. All available 31 single-nucleotide polymorphisms (SNP) in and near *LEPR* gene were selected for the analyses, including two commonly investigated SNPs, Arg109Lys (rs1137100) and Arg223Gln (rs1137101). SNPs in leptin gene region (LEP) were not included in the list of SNPs genotyped using HumanHap300 chip and therefore were not available for the analyses.

In the Split sample, plasma for CRP levels was stored in a freezer at − 80 °C and transported frozen to a Medical Research Council Human Genetics, Edinburgh. Highly sensitive CRP was measured by enzyme-linked immunosorbent assay (ELISA ; R&D systems, Minneapolis, MN, USA) using calibrants and reagents provided by the manufacturer. DNA was extracted from blood samples using Nucleon DNA purification kits (Tepnel). Genotyping was performed using the Illumina's Sentrix HumanHap370CNV Genotyping BeadChip (v1) comprising > 370 000 markers. Two SNPs that were included on the HumanHap300 chip, rs1171269 and rs10493384, were not on the HumanHap370CNV chip. All three biochemical laboratories are internationally accredited for performing the conducted analyses.

Cardiovascular risk phenotypes analyzed in this study were body mass index (BMI), waist-to-hip-ratio, waist circumference (WC), total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, fibrinogen and C-reactive protein level. Anthropometric measurements were taken according to the standard International Biological Programme (IBP) Protocol.[27] BMI was calculated as weight in kilograms divided by squared height in meters ($kg\,m^{-2}$). After quality control of genotyping data, Arg109Lys (rs1137100) was excluded from analyses in the Vis sample due to high genotyping failure rate (0.1997). Hardy–Weinberg equilibrium and linkage disequilibrium (LD) structures in *LEPR* genomic region were evaluated using Haploview software. The same software was used to define haplotype blocks. The rs17448446 did not conform to Hardy–Weinberg equilibrium in the Vis sample. As this SNP is the farthest from the end of the coding region and was not a part of a haplotype block, it was excluded from further analyses in both the Vis and the inland sample. Association of different genotypes of the individual polymorphisms with the selected cardiovascular risk phenotypes was tested by linear regression controlling for sex and age. The differences in mean values of quantitative biochemical variables were tested using *t*-test for independent samples. As mean age significantly differed between the Vis and the inland sample in both sexes, we validated the results by testing age-adjusted residuals obtained with linear regression. Statistical analyses were performed using IBM SPSS Statistics Version 22, VALICON, Zagreb, Croatia. As individual SNPs selected for the analyses could be in LD and therefore not independent, application of Bonferroni correction for multiple testing would probably result in overcorrection for the inflated false-positive rate and significantly reduce the power. To overcome this problem, we applied a correction for multiple testing based on spectral decomposition of matrices of pair-wise LD between SNPs, implemented in SNPSpD (http://genepi.qimr.edu.au/general/daleN/SNPSpD/).[28,29] Unphased version 3.0.13 software was used for haplotype association analysis.[30] This analysis is based on a retrospective likelihood, which is the probability of observing the parental and child genotypes, given the trait values of all the children in a nuclear family. Unrelated subjects were treated as children of missing parents. Correction for multiple testing was conducted through permutation tests (1000 permutations).

## RESULTS

The clinical characteristics of the Vis and the Split sample are described and compared in Table 1. The given results confirm the presumption on the elevated values of the CVD-risk-associated anthropometric variables in the Vis sample but only in women: both BMI and WC were significantly higher in women from Vis than in the women from Split. Mean WC values in both sex inhabitants of Vis and in females from Split were above cutoffs according to both Qiao and Nyamdorj study and the International Diabetes Federation (IDF) consensus for definition of the central obesity.[31,32]

Most of the biochemical parameters were also elevated according to IDF and Adult Treatment Panel III Criteria for the Metabolic Syndrome.[33] Dyslipidemia was more pronounced in the Split sample than in the Vis sample in both sexes, and the differences were significant even after the age adjustment.

SNPs were grouped into eight blocks according to the LD structure in the Vis sample and in seven blocks in the inland sample (Figures 1 and 2). Individual SNPs, one in the haplotype block 1 located in the promoter region (rs3806318), and others in the haplotype block 4 in intron 2 region (rs1171279) and in blocks 7 and 8 in the 3′ region (rs2186245 and rs7539471) of the *LEPR* gene, were associated with serum lipids (Table 2). SNPs in the 3′ region of the gene correlated with fibrinogen (rs1892534, rs12022410, rs2211651, rs2889195, rs7539471, rs4291477) and with CRP levels (rs2211651, rs2889195, rs12753193, rs7539471). However, after correction for multiple testing with experiment-wide multiple testing significance threshold determined using SNPSpD set at 0.00314, only rs4291477 showed significant association ($P = 0.00282$) with fibrinogen and rs7539471 marginally significant ($P = 0.00379$) association with HDL. The minor G-allele carriers of rs4291477 had higher fibrinogen, and the major G-allele carriers of rs7539471 had lower HDL levels.

Although none of the above-mentioned results replicated in the inland sample, rs10493384, which is in the same haplotype block as rs4291477 and rs7539477, showed marginally significant association with triglyceride levels ($P = 0.006$, threshold determined using SNPSpD = 0.00252). The minor T-allele carriers had higher triglyceride levels.

**Table 1 Comparison of clinical parameters of the Vis and inland (Split) population**

| *Variables* | *Male* | | P-*value* | *Female* | | P-*value* |
|---|---|---|---|---|---|---|
| | *Vis* | *Split* | | *Vis* | *Split* | |
| *Sample size (N)* | 400 | 213 | | 586 | 286 | |
| Age (years) | 56.25±14.90 | 47.92±15.51 | <0.001 | 56.33±16.04 | 49.88±16.04 | <0.001 |
| BMI (kg m$^{-2}$) | 27.57±3.73 | 28.10±3.86 | NS* | 27.16±4.65 | 26.06±4.22 | <0.001* |
| Waist circumference (mm) | 985.23±99.80 | 971.76±110.92 | NS | 939.94±128.60 | 863.95±107.94 | <0.001* |
| Total cholesterol (mmol l$^{-1}$) | 5.06±1.01 | 5.83±1.31 | <0.001* | 5.13±0.95 | 5.92±1.22 | <0.001* |
| HDL (mmol l$^{-1}$) | 1.11±0.17 | 1.20±0.26 | <0.001* | 1.11±0.15 | 1.51±0.32 | <0.001* |
| LDL (mmol l$^{-1}$) | 3.22±0.98 | 3.82±1.09 | <0.001* | 3.24±0.90 | 3.82±1.05 | <0.001* |
| Triglycerids (mmol l$^{-1}$) | 1.69±0.99 | 1.98±2.10 | <0.05* | 1.72±1.00 | 1.28±0.66 | <0.001* |
| Fibrinogen (g l$^{-1}$) | 3.64±0.98 | 3.63±1.17 | NS | 3.81±1.00 | 3.99±1.15 | <0.05* |
| CRP (mg l$^{-1}$) | 2.62±4.71 | 2.63±2.63 | NS | 2.47±3.58 | 2.49±2.76 | NS |

Abbreviations: BMI, body mass index; CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NS, not significant. Asterisks (*) relate to the significant differences obtained from the age-adjusted residuals calculated using linear regression with age and age squared as single predictors.

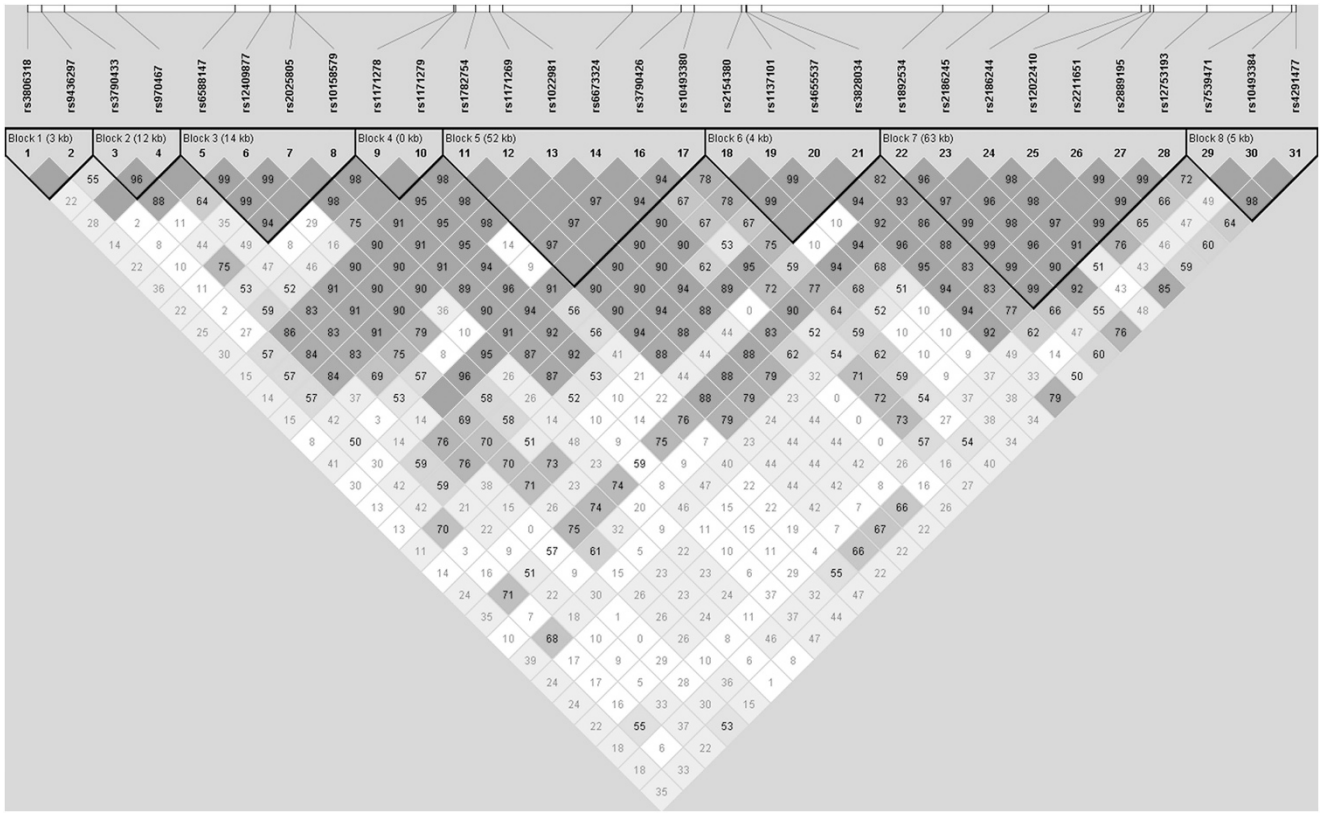


**Figure 1** Haploview-generated graphical representation of LD in *LEPR* gene region in the Vis sample. Thirty-one SNP spanning from position 65885357 to 66191075 according to GRCh37.p10 assembly were grouped into eight blocks as indicated by triangles in the picture. Length of each block is provided in kilobases (kb), and pairwise LD (D′) is given for each SNP combination (showed in box). The color code in the haploview plot follows the standard color scheme for Haploview: white, ID′I<1, LOD<2; shades of pink/red, ID′I<1, LOD⩾2; blue, I D′I=1, LOD<2; red, ID′I=1, LOD⩾2. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Haplotype association results were as following: the strongest association signal with any of the tested CVD risk phenotypes in all the eight LD blocks in the Vis sample was shown by the haplotype A-C-A-A-G-A in the fifth block with WC ($P=7.085\times10^{-22}$), although none of its six SNPs (rs1782754, rs1171269, rs1022981, rs6673324, rs3790426 and rs10493380) was individually associated with WC (Table 3). However, after permutation testing, neither this result nor the other association signals proved to be statistically significant.

In addition, the eighth block, although it included the individually significant rs4291477, showed no association with any of the tested phenotypes. Haplotype A-A-A-A in block four in the inland data (rs1782754, rs1022981, rs6673324 and rs1137100) was also nominally associated with WC, $P=5.496\ \text{x}10^{-144}$. This haplotype block consisted of three SNPs that were also part of haplotype block five in the Vis data and spanned approximately the same region. This result did not survive permutation testing, either (Table 4).

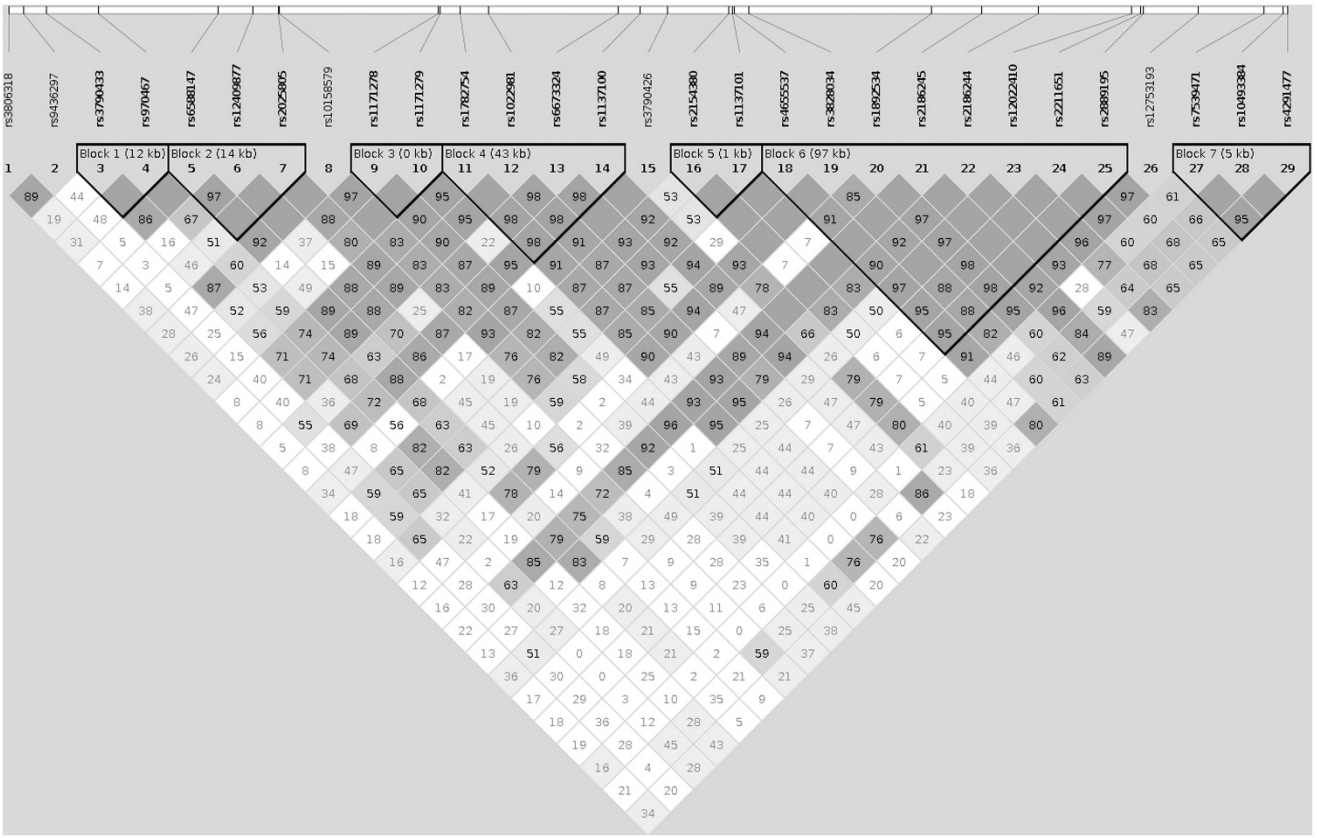


**Figure 2** Haploview-generated graphical representation of LD in *LEPR* gene region in Split (inland) sample. Twenty-nine SNPs spanning from position 65885357 to 66191075 according to GRCh37.p10 assembly were grouped into seven blocks as indicated by triangles in the picture. Length of each block is provided in kilobases (kb), and pairwise LD (D′) is given for each SNP combination (showed in box). The color code in the Haploview plot follows the standard color scheme for Haploview: white, ID′I < 1, LOD < 2; shades of pink/red, ID′I < 1, LOD ⩾ 2; blue, I D′I = 1, LOD < 2; red, ID′I = 1, LOD ⩾ 2. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

To allow for more accurate phasing of the haplotypes and to further evaluate possible correlation of haplotypes with the studied phenotypes, we tested the subsample of only family-related individuals in the Vis sample (number of nuclear families $N = 366$, number of individuals $N = 576$). None of the weak association signals detected in the subsample survived permutation testing.

## DISCUSSION

*LEP* and *LEPR* are among the most frequently studied genes regarding their impact on obesity and obesity-related phenotypes. Owing to the strong indication of link between leptin and CVDs, the association of polymorphisms in leptin gene and/or *LEPR* with CVDs or CVD risk factors is being investigated intensively.[21,34–37] However, most of these researchers focused on the influence of individual polymorphisms on the studied phenotype, and only a few investigated the role of haplotype blocks as well.

To take the advantage of strong LD in and near *LEPR* gene and well-defined haplotype blocks, we investigated the association of both individual polymorphisms as well as the association of eight haplotype blocks spanning wide *LEPR* gene region with phenotypes of interest. Several of the 31 investigated SNPs showed individual association signals: SNPs located in the promoter region (rs3806318), intron 2 region (rs1171279) and 3′ region (rs2186245, rs7539471) of the *LEPR* gene were associated with serum lipids. However, after correction for multiple testing, only rs7539471 remained marginally associated with HDL cholesterol. Neither was this, nor were the other three mentioned SNPs previously reported as being associated with serum lipid levels. In some studies, two coding polymorphisms in *LEPR* gene, Asn656Lys (rs1805094) and Gln223Arg (rs1137101), were reported as significant predictors of serum lipids levels, whereas in other studies no association or linkage of any of the investigated *LEPR* polymorphisms with serum lipids was found.[37–44] The Gln223Arg (rs1137101) polymorphism was tested in this study but showed no association with lipid levels.

The strongest individual associations of SNPs in and near 3′ region of the *LEPR* gene were with fibrinogen (rs1892534, rs12022410, rs2211651, rs2889195, rs7539471, rs4291477) and CRP levels (rs2211651, rs2889195, rs12753193, rs7539471). Among these, rs4291477 was found to be significantly associated with fibrinogen even after applying correction for multiple testing. rs4291477 is located 87,899 bases away from the stop codon of the 3′ region of the *LEPR* gene and to this date there is no information on the possible functional effect of this SNP. Still, as this particular association with fibrinogen was not found previously, but, interestingly, Ridker *et al.*[45] found the association of the same locus with CRP levels, we presume that this polymorphism has an important role in the inflammation process. Moreover, that group reported that all four *LEPR* SNPs linked with CRP, which did not survive the correction for multiple testing in our sample, were significantly associated with CRP in Women's Genome Health Study sample. In addition to that, two SNPs that in

**Table 2 SNPs nominally associated with CVD risk phenotypes**

| *SNP* | *Minor allele* | *MAF* | *Phenotype* | *Beta* | *S.e.* | P-*value* |
|---|---|---|---|---|---|---|
| *(a) Vis* | | | | | | |
| rs3806318 | G | 0.214 | Total cholesterol | −0.128 | 0.063 | 0.042 |
| rs3806318 | G | 0.214 | LDL cholesterol | −0.120 | 0.061 | 0.048 |
| rs1171279 | T | 0.251 | Fibrinogen | −0.125 | 0.054 | 0.021 |
| rs1892534 | T | 0.357 | Fibrinogen | −0.103 | 0.047 | 0.028 |
| rs2186245 | T | 0.157 | HDL cholesterol | −0.025 | 0.010 | 0.016 |
| rs12022410 | A | 0.428 | Fibrinogen | 0.088 | 0.045 | 0.051 |
| rs2211651 | T | 0.357 | Fibrinogen | −0.112 | 0.047 | 0.018 |
| rs2211651 | T | 0.357 | CRP | −0.446 | 0.195 | 0.022 |
| rs2889195 | T | 0.357 | Fibrinogen | 0.105 | 0.047 | 0.024 |
| rs2889195 | T | 0.357 | CRP | 0.422 | 0.197 | 0.032 |
| rs12753193 | G | 0.342 | CRP | −0.404 | 0.197 | 0.041 |
| rs7539471 | A | 0.353 | CRP | 0.470 | 0.204 | 0.021 |
| rs7539471 | A | 0.353 | HDL cholesterol | −0.022 | 0.008 | 0.004 |
| rs7539471 | A | 0.353 | Fibrinogen | 0.106 | 0.048 | 0.028 |
| rs4291477 | G | 0.494 | Fibrinogen | −0.134 | 0.045 | 0.003* |
| *(b) Split* | | | | | | |
| rs3806318 | G | 0.307 | Triglycerides | 0.224 | 0.104 | 0.031 |
| rs2025805 | T | 0.45 | CRP | −0.354 | 0.170 | 0.038 |
| rs10158579 | C | 0.123 | CRP | 0.577 | 0.258 | 0.026 |
| rs1171278 | T | 0.171 | CRP | −0.454 | 0.222 | 0.042 |
| rs1137100 | G | 0.261 | HDL cholesterol | −0.048 | 0.022 | 0.027 |
| rs1137101 | G | 0.492 | HDL cholesterol | −0.035 | 0.018 | 0.042 |
| rs2186245 | T | 0.174 | HDL cholesterol | 0.050 | 0.025 | 0.042 |
| rs2186244 | A | 0.182 | HDL cholesterol | 0.054 | 0.023 | 0.02 |
| rs10493384 | T | 0.17 | Triglycerides | −0.348 | 0.126 | 0.006 |

Abbreviations: CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein. The alleles are reported in forward orientation with respect to the genome. Table shows unadjusted *P*-values, asterisk (*) relates to associations significant after correction for multiple testing.

**Table 3 Vis sample: the presentation of nominal associations (unadjusted *P*-values) of haplotypes with the investigated CVD risk phenotypes.**

| *Phenotype* | *Haplotype block No.* | *Haplotype alleles* | P-*value* |
|---|---|---|---|
| BMI | 1 | A-T | 0.003 |
| | 5 | A-C-A-A-G-G | 0.027 |
| | 6 | A-A-G-T | 0.016 |
| Waist circumference | 5 | A-C-A-A-G-A | $7.085\times10^{-22}$* |
| | | G-T-G-A-G-A | $2.215\times10^{-6}$ |
| Total cholesterol | 7 | A-A-C-G-T-T-G | 0.005 |
| HDL cholesterol | 7 | A-A-C-G-T-T-G | $0.417\times10^{-5}$ |
| LDL cholesterol | 5 | A-C-A-G-G-G | 0.046 |
| | 6 | G-G-G-T | $1.269\times10^{-8}$ |
| | 7 | A-A-C-G-T-T-G | 0.001 |
| Triglycerides | 6 | A-A-G-T | 0.055 |
| Fibrinogen | 7 | A-G-T-A-T-T-A | 0.065 |
| CRP | 5 | A-C-A-A-G-G | 0.066 |
| | 8 | A-G-G | 0.084 |

Abbreviations: BMI, body mass index; CVD, cardiovascular disease; CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein. None of the haplotype associations remained significant after permutation testing.
*Adjusted *P*-value from permutation test: 0.0979.

our sample lost significance for their association with fibrinogen after the correction (rs1892534, rs12022410), significantly associated with CRP levels in Ridker's sample. The rs1892534 was found to statistically significantly associate with CRP levels also by Curocichin *et al.*[46] and by Wu *et al.*[47] The association of *LEPR* SNP rs12753193 with CRP levels, which in our sample did not survive the correction, was found also by Sabatti *et al.*,[48] while Marzi *et al.*[49] reported its association with both CRP and fibrinogen levels. Several other *LEPR* polymorphic loci were also found to be associated with CRP levels, and one study reported association of haplotype block that included three commonly investigated *LEPR* missense SNPs, Lys109Arg (rs1137100), Gln223Arg (rs1137101) and Asn656Lys (rs1805094), with both CRP and fibrinogen serum levels.[21,34,45,50]

CRP and fibrinogen are involved in inflammatory response as acute-phase reactants, proteins whose serum levels change in response to high cytokine levels in acute, but also in chronic inflammatory state that is often present in obesity, and has a key role in atherosclerosis and CVDs.[10,51] Therefore, it was proposed that proinflammatory state, characterized by elevated CRP levels, and prothrombotic state, characterized by elevated fibrinogen levels, may be metabolically interconnected.[33] In our study, fibrinogen is associated with individual SNP in 3′ region of the *LEPR* gene, which codes for the variable intracellular signaling part of the LepR.[52] The quest for genes that determine variance in obesity-related traits (including BMI, WC, waist-to-hip ratio and so on) has been extensive: the *LEPR* polymorphic loci contribute to this variance as well.[53–57] Although SNPs Arg109Lys (rs1137100) and Arg223Gln (rs1137101) were reported as being associated with obesity or obesity-related phenotypes in some studies, other researchers found no association, which could possibly be attributed to inadequate power because of small sample sizes.[58–68] However, a systematic meta-analysis of linkage and association of the three most commonly investigated *LEPR* polymorphisms located in

**Table 4 Split sample: the presentation of nominal associations (unadjusted *P*-values) of haplotypes with the investigated CVD risk phenotypes**

| *Phenotype* | *Haplotype block No.* | *Haplotype alleles* | P-*value* |
|---|---|---|---|
| Waist circumference | 4 | A-A-A-A | $5.496\times10^{-144*}$ |
| | | A-A-G-A | $1.515\times10^{-41}$ |
| | | G-G-A-A | $1.245\times10^{-12}$ |
| | | G-G-A-G | $2.329\times10^{-12}$ |
| | 7 | G-G-G | $3.958\times10^{-12}$ |
| HDL cholesterol | 4 | A-A-G-A | 0.04117 |
| | | G-G-A-G | 0.02481 |
| | 6 | G-G-A-G-A-G-A-A | 0.0379 |
| | | G-G-G-G-G-G-C-G | 0.009349 |
| Fibrinogen | 6 | G-A-A-A-G-G-A-A | 0.04007 |
| | 7 | G-G-G | 0.02405 |
| CRP | 5 | A-G-G | $4.283\times10^{-15}$ |

Abbreviations: CVD, cardiovascular disease; CRP, C-reactive protein; HDL, high-density lipoprotein. None of the haplotype associations remained significant after permutation testing.
*Adjusted *P*-value from permutation test: 0.1062.

the coding region of the gene, Arg109Lys (rs1137100), Arg223Gln (rs1137101) and Lys656Asn (rs1805094), with BMI and WC showed no evidence of association.[69] Our results support the later findings; we investigated two out of three SNPs in Heo's study, Arg109Lys (rs1137100) and Arg223Gln (rs1137101), and none of them showed association with any of the investigated obesity-related phenotypes.

Although our sample comprises more than one-third of the adult inhabitants of the island of Vis, however, sample size is quite modest for a study of this design. In addition, despite the fact that shared ecological environment does provide an advantage in the association studies, it does not exclude the possibility of uncontrolled confounding variables. Our results did not replicate in the only available independent sample with a similar ecological environment. However, one should have in mind that our results may be affected by inadequate statistical power resulting from a small inland sample size ($N=499$). Therefore, we propose that results regarding association of individual SNPs with lipids, fibrinogen and CRP, as well as the association of haplotypes with WC, should be further investigated in a metapopulation of Croatian island isolates, which all show surprisingly high prevalence of cardiovascular risk phenotypes.[70,71]

To conclude, regardless of the strong LD between individual SNPs in *LEPR* gene region and clear haplotype block structure, haplotype association analysis did not provide any advantage over analysis of individual SNPs. Analyzed haplotypes spanning wide *LEPR* gene region were not found to be significant predictors of cardiovascular risk phenotypes, showing only marginal association with waist circumference. However, in the Vis population rs4291477 was associated with fibrinogen levels. Although this association did not replicate in additional, inland sample, which might be due to the small sample size, it does have a potential for use in preventive medicine, specifically, in the early detection of people with predisposition for inflammatory and collagene vascular diseases.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

We are deeply grateful to the people of Vis for their kindness and interest for participation in the study. Fieldwork and genotyping were done within the scope of the project 'Genetics of complex traits and diseases in Croatian genetic isolates', we thank the collaborators on these project from Medical Research Council-Human Genetic Unit and University Medical School, University of Edinburgh for their continuos support. We specially thank Dr. Caroline Hayward and Dr. Ozren Polašek for sharing the Split data and all the helpful suggestions. This work was supported by the Croatian Ministry of Science, Education, and Sports of the Republic of Croatia (through grant 108-1080315-0302 awarded to I. Rudan, grant 196-1962766-2747 awarded to N. Smolej Narančić, grant 196-1962766-2751 awarded to P. Rudan, and grant 196-1962766-2763 awarded to B. Janićijević) and by the Medical Research Council UK through grants awarded to H. Campbell and I. Rudan.

*Author contributions:* Željka Tomas, Nina Smolej Narančić and Tatjana Škarić-Jurić developed the study design. Nina Smolej Narančić, Tatjana Škarić-Jurić, Marijana Peričić Salihović conducted field research and performed the data collection and quality control. Željka Tomas, Matea Zajc Petranović and Ana Barešić performed the statistical analyses. Željka Tomas wrote the first draft of the paper. All authors contributed to the writing of the final version of the paper.

1 Mendis, S., Puska, P. & Norrving, B. *Global Atlas on Cardiovascular Disease Prevention and Control* (World Health Organization, Geneva, Switzerland, 2011).
2 Gami, A. S., Witt, B. J., Howard, D. E., Erwin, P. J., Gami, L. A., Somers, V. K. *et al.* Metabolic syndrome and risk of incident cardiovascular events and death: a systematic review and meta-analysis of longitudinal studies. *J. Am. Coll. Cardiol.* **49**, 403–414 (2007).
3 Lee, G. H., Proenca, R., Montez, J. M., Carroll, K. M., Darvishzadeh, J. G., Lee, J. I. *et al.* Abnormal splicing of the leptin receptor in diabetic mice. *Nature* **379**, 632–635 (1996).
4 Tartaglia, L. A. The leptin receptor. *J. Biol. Chem.* **272**, 6093–6096 (1997).
5 Maness, L. M., Banks, W. A. & Kastin, A. J. Persistence of blood-to-brain transport of leptin in obese leptin-deficient and leptin receptor-deficient mice. *Brain Res.* **873**, 165–167 (2000).
6 Heymsfield, S. B., Greenberg, A. S., Fujioka, K., Dixon, R. M., Kushner, R., Hunt, T. *et al.* Recombinant leptin for weight loss in obese and lean adults: a randomized, controlled, dose-escalation trial. *JAMA* **282**, 1568–1575 (1999).
7 Bell-Anderson, K. S. & Bryson, J. M. Leptin as a potential treatment for obesity: progress to date. *Treat. Endocrinol.* **3**, 11–18 (2004).
8 Münzberg, H., Björnholm, M., Bates, S. H. & Myers, M. G. Jr. Leptin receptor action and mechanisms of leptin resistance. *Cell Mol. Life. Sci.* **62**, 642–652 (2005).
9 Lam, Q. L. & Lu, L. Role of leptin in immunity. *Cell Mol. Immunol.* **4**, 1–13 (2007).
10 Burgos-Ramos, E., Sackmann-Sala, L., Baquedano, E., Cruz-Topete, D., Barrios, V., Argente, J. *et al.* Central leptin and insulin administration modulates serum cytokine- and lipoprotein-related markers. *Metabolism* **61**, 1646–1657 (2012).
11 Konstantinides, S., Schafer, K. & Loskutoff, D. J. The prothrombotic effects of leptin possible implications for the risk of cardiovascular disease in obesity. *Ann. N. Y. Acad. Sci.* **947**, 134–142 (2001).
12 Bodary, P. F., Westrick, R. J., Wickenheiser, K. J., Shen, Y. & Eitzman, D. T. Effect of leptin on arterial thrombosis following vascular injury in mice. *JAMA* **287**, 1706–1709 (2002).
13 Tsatsoulis, A., Mantzaris, M. D., Bellou, S. & Andrikoula, M. Insulin resistance: an adaptive mechanism becomes maladaptive in the current environment - an evolutionary perspective. Review. *Metabolism* **62**, 622–633 (2013).
14 Soderberg, S., Ahren, B., Jansson, J. H., Johnson, O., Hallmans, G., Asplund, K. *et al.* Leptin is associated with increased risk of myocardial infarction. *J. Intern. Med.* **246**, 409–418 (1999).
15 Wallace, A. M., McMahon, A. D., Packard, C. J., Kelly, A., Shepherd, J., Gaw, A. *et al.* Plasma leptin and the risk of cardiovascular disease in the west of Scotland coronary prevention study (WOSCOPS). *Circulation* **104**, 3052–3056 (2001).
16 Thøgersen, A. M., Söderberg, S., Jansson, J. H., Dahlén, G., Boman, K., Nilsson, T. K. *et al.* Interactions between fibrinolysis, lipoproteins and leptin related to a first myocardial infarction. *Eur. J. Cardiovasc. Prev. Rehabil.* **11**, 33–40 (2004).
17 Wallerstedt, S. M., Eriksson, A. L., Niklason, A., Ohlsson, C. & Hedner, T. Serum leptin and myocardial infarction in hypertension. *Blood Press* **13**, 243–246 (2004).
18 Wolk, R., Berger, P., Lennon, R. J., Brilakis, E. S., Johnson, B. D. & Somers, V. K. Plasma leptin and prognosis in patients with established coronary atherosclerosis. *J. Am. Coll. Cardiol.* **44**, 1819–1824 (2004).
19 Reilly, M. P., Iqbal, N., Schutta, M., Wolfe, M. L., Scally, M., Localio, A. R. *et al.* Plasma leptin levels are associated with coronary atherosclerosis in type 2 diabetes. *J. Clin. Endocrinol. Metab.* **89**, 3872–3878 (2004).
20 Hamnvik, O. P., Liu, X., Petrou, M., Gong, H., Chamberland, J. P., Kim, E. H. *et al.* Soluble leptin receptor and leptin are associated with baseline adiposity and metabolic risk factors, and predict adiposity, metabolic syndrome, and glucose levels at 2-year follow-up: the Cyprus Metabolism Prospective Cohort Study. *Metabolism* **60**, 987–993 (2011).
21 Zhang, Y. Y., Gottardo, L., Mlynarski, W., Frazier, W., Nolan, D., Duffy, J. *et al.* Genetic variability at the leptin receptor (*LEPR*) locus is a determinant of plasma fibrinogen and C-reactive protein levels. *Atherosclerosis* **191**, 121–127 (2007).

22 Dehghan, A., Dupuis, J., Barbalic, M., Bis, J. C., Eiriksdottir, G., Lu, C. *et al.* Meta-analysis of genome-wide association studies in >80 000 subjects identifies multiple loci for C-reactive protein levels. *Circulation* **123**, 731–738 (2011).
23 Clark, A. G. The role of haplotypes in candidate gene studies. *Genet. Epidemiol.* **27**, 321–333 (2004).
24 Barbalić, M., Narančić, N. S., Škarić-Jurić, T., Salihović, M. P., Martinović Klarić, I., Lauc, L. B. *et al.* A quantitative trait locus for SBP maps near KCNB1 and PTGIS in a population isolate. *Am. J. Hypertens.* **22**, 663–668 (2009).
25 Missoni, S. Metabolic syndrome among the inhabitants of the island of Vis. *Coll. Antropol.* **33**, 1281–1287 (2009).
26 Vitart, V., Rudan, I., Hayward, C., Gray, N. K., Floyd, J., Palmer, C. N. *et al.* SLC2A9 is a newly identified urate transporter influencing serum urate concentration, urate excretion and gout. *Nat. Genet.* **40**, 437–442 (2008).
27 Weiner, J. S. & Lourie, J. A. *Practical Human Biology* (Academic Press, London, UK, 1981).
28 Nyholt, D. R. A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. *Am. J. Hum. Genet.* **74**, 765–769 (2004).
29 Li, J. & Ji, L. Adjusting multiple testing in multilocus analyses using the eigenvalues of a correlation matrix. *Heredity* **95**, 221–227 (2005).
30 Dudbridge, F. Likelihood-based association analysis for nuclear families and unrelated subjects with missing genotype data. *Hum. Hered.* **66**, 87–98 (2008).
31 Qiao, Q. & Nyamdorj, R. The optimal cutoff values and their performance of waist circumference and waist-to-hip ratio for diagnosing type II diabetes. *Eur. J. Clin. Nutr.* **64**, 23–29 (2010).
32 Alberti, K. G., Zimmet, P. & Shaw, J. Metabolic syndrome–a new world-wide definition. A Consensus Statement from the International Diabetes Federation. *Diabet. Med.* **23**, 469–480 (2006).
33 Grundy, S. M., Cleeman, J. I., Merz, C. N., Brewer, H. B. Jr, Clark, L. T., Hunninghake, D. B. *et al.* Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III guidelines. *Circulation* **110**, 227–239 (2004).
34 Elliott, P., Chambers, J. C., Zhang, W., Clarke, R., Hopewell, J. C., Peden, J. F. *et al.* Genetic loci associated with C-reactive protein levels and risk of coronary heart disease. *JAMA* **302**, 37–48 (2009).
35 Sober, S., Org, E., Kepp, K., Juhanson, P., Eyheramendy, S., Gieger, C. *et al.* Targeting 160 candidate genes for blood pressure regulation with a genome-wide genotyping array. *PLoS ONE* **4**, e6034 (2009).
36 Phillips, C. M., Goumidi, L., Bertrais, S., Field, M. R., Ordovas, J. M., Cupples, L. A. *et al.* Leptin receptor polymorphisms interact with polyunsaturated fatty acids to augment risk of insulin resistance and metabolic syndrome in adults. *J. Nutr.* **140**, 238–244 (2010).
37 Jackson, K. G., Delgado-Lista, J., Gill, R., Lovegrove, J. A., Williams, C. M., López-Miranda, J. *et al.* The leptin receptor Gln223Arg polymorphism (rs1137101) mediates the postprandial lipaemic response, but only in males. *Atherosclerosis* **225**, 135–141 (2012).
38 Angel-Chávez, L. I., Tene-Pérez, C. E. & Castro, E. Leptin receptor gene K656N polymorphism is associated with low body fat levels and elevated high-density cholesterol levels in Mexican children and adolescents. *Endocr. Res.* **37**, 124–134 (2012).
39 Popruk, S., Tungtrongchitr, R., Petmitr, S., Pongpaew, P., Harnroongroj, T., Pooudong, S. *et al.* A. Leptin, soluble leptin receptor, lipid profiles, and *LEPR* gene polymorphisms in Thai children and adolescents. *Int. J. Vitam. Nutr. Res.* **78**, 9–15 (2008).
40 van der Vleuten, G. M., Kluijtmans, L. A., Hijmans, A., Blom, H. J., Stalenhoef, A. F., de Graaf, J. *et al.* The Gln223Arg polymorphism in the leptin receptor is associated with familial combined hyperlipidemia. *Int. J. Obes.* **30**, 892–898 (2006).
41 Ukkola, O., Tremblay, A., Després, J. P., Chagnon, Y. C., Campfield, L. A. & Bouchard, C. Leptin receptor Gln223Arg variant is associated with a cluster of metabolic abnormalities in response to long-term overfeeding. *J. Intern. Med.* **248**, 435–439 (2000).
42 Popko, K., Gorska, E., Wasik, M., Stoklosa, A., Pływaczewski, R., Winiarska, M. *et al.* Frequency and distribution of leptin receptor polymorphism in obstructive sleep APNEA patients. *J. Physiol. Pharmacol.* **58**(Suppl 5 (Pt 2)), 551–561 (2007).
43 Ogawa, T., Hirose, H., Yamamoto, Y., Nishikai, K., Miyashita, K., Nakamura, H. *et al.* Relationships between serum soluble leptin receptor level and serum leptin and adiponectin levels, insulin resistance index, lipid profile, and leptin receptor gene polymorphisms in the Japanese population. *Metabolism* **53**, 879–885 (2004).
44 Souren, N. Y., Zeegers, M. P., Janssen, R. G., Steyls, A., Gielen, M., Loos, R. J. *et al.* Anthropometry, carbohydrate and lipid metabolism in the East Flanders Prospective Twin Survey: linkage of candidate genes using two sib-pair based variance components analyses. *Twin. Res. Hum. Genet.* **11**, 505–516 (2008).
45 Ridker, P. M., Pare, G., Parker, A., Zee, R. Y., Danik, J. S., Buring, J. E. *et al.* Loci related to metabolic-syndrome pathways including *LEPR, HNF1A, IL6R*, and *GCKR* associate with plasma C-reactive protein: the Women's Genome Health Study. *Am. J. Hum. Genet.* **82**, 1185–1192 (2008).
46 Curocichin, G., Wu, Y., McDade, T. W., Kuzawa, C. W., Borja, J. B., Qin, L. *et al.* Single-nucleotide polymorphisms at five loci are associated with C-reactive protein levels in a cohort of Filipino young adults. *J. Hum. Genet.* **56**, 823–827 (2011).
47 Wu, Y., McDade, T. W., Kuzawa, C. W., Borja, J., Li, Y., Adair, L. S. *et al.* Genome-wide association with C-reactive protein levels in CLHNS: evidence for the CRP and HNF1A loci and their interaction with exposure to a pathogenic environment. *Inflammation* **35**, 574–583 (2012).
48 Sabatti, C., Service, S. K., Hartikainen, A. L., Pouta, A., Ripatti, S., Brodsky, J. *et al.* Genome-wide association analysis of metabolic traits in a birth cohort from a founder population. *Nat. Genet.* **41**, 35–46 (2009).
49 Marzi, C., Albrecht, E., Hysi, P. G., Lagou, V., Waldenberger, M., Tönjes, A. *et al.* Genome-wide association study identifies two novel regions at 11p15.5-p13 and 1p31 with major impact on acute-phase serum amyloid A. *PLoS Genet.* **6**, e1001213 (2010).
50 Reiner, A. P., Beleza, S., Franceschini, N., Auer, P. L., Robinson, J. G., Kooperberg, C. *et al.* Genome-wide association and population genetic analysis of C-reactive protein in African American and Hispanic American women. *Am. J. Hum. Genet.* **91**, 502–512 (2012).
51 Libby, P. Inflammation in atherosclerosis. *Nature* **420**, 868–874 (2002).
52 Steiner, A. A. & Romanovsky, A. A. Leptin: at the crossroads of energy balance and systemic inflammation. *Prog. Lipid Res.* **46**, 89–107 (2007).
53 Loos, R. J. Genetic determinants of common obesity and their value in prediction. *Best Pract. Res. Clin. Endocrinol. Metab.* **26**, 211–226 (2012).
54 Bradfield, J. P., Taal, H. R., Timpson, N. J., Scherag, A., Lecoeur, C., Warrington, N. M. *et al.* A genome-wide association meta-analysis identifies new childhood obesity loci. *Nat. Genet.* **44**, 526–531 (2012).
55 Traurig, M. T., Perez, J. M., Ma, L., Bian, L., Kobes, S., Hanson, R. L. *et al.* Variants in the *LEPR* gene are nominally associated with higher BMI and lower 24-h energy expenditure in Pima Indians. *Obesity* **20**, 2426–2430 (2012).
56 Lombard, Z., Crowther, N. J., van der Merwe, L., Pitamber, P., Norris, S. A., Ramsay, M. *et al.* Appetite regulation genes are associated with body mass index in black South African adolescents: a genetic association study. *BMJ Open* **2**: pii e000873 (2012).
57 Wheeler, E., Huang, N., Bochukova, E. G., Keogh, J. M., Lindsay, S., Garg, S. *et al.* Genome-wide SNP and CNV analysis identifies common and low-frequency variants associated with severe early-onset obesity. *Nat. Genet.* **45**, 513–517 (2013).
58 Chagnon, Y. C., Chung, W. K., Pérusse, L., Chagnon, M., Leibel, R. L., Bouchard, C. *et al.* Linkages and associations between the leptin receptor (*LEPR*) gene and human body composition in the Québec Family Study. *Int. J. Obes. Relat. Metab. Disord.* **23**, 278–286 (1999).
59 Yiannakouris, N., Yannakoulia, M., Melistas, L., Chan, J. L., Klimis-Zacas, D., Mantzoros, C. S. *et al.* The Q223R polymorphism of the leptin receptor gene is significantly associated with obesity and predicts a small percentage of body weight and body composition variability. *J. Clin. Endocrinol. Metab.* **86**, 4434–4439 (2001).
60 Stefan, N., Vozarova, B., Del Parigi, A., Ossowski, V., Thompson, D. B., Hanson, R. L. *et al.* The Gln223Arg polymorphism of the leptin receptor in Pima Indians: influence on energy expenditure, physical activity and lipid metabolism. *Int. J. Obes. Relat. Metab. Disord.* **26**, 1629–1632 (2002).
61 Wauters, M., Mertens, I., Chagnon, M., Rankinen, T., Considine, R. V., Chagnon, Y. C. *et al.* Polymorphisms in the leptin receptor gene, body composition and fat distribution in overweight and obese women. *Int. J. Obes. Relat. Metab. Disord.* **25**, 714–720 (2001).
62 Murugesan, D., Arunachalam, T., Ramamurthy, V. & Subramanian, S. Association of polymorphisms in leptin receptor gene with obesity and type 2 diabetes in the local population of Coimbatore. *Indian J. Hum. Genet.* **16**, 72–77 (2010).
63 Tabassum, R., Mahendran, Y., Dwivedi, O. P., Chauhan, G., Ghosh, S., Marwaha, R. K. *et al.* Common variants of *IL6, LEPR*, and *PBEF1* are associated with obesity in Indian children. *Diabetes* **61**, 626–631 (2012).
64 Boumaiza, I., Omezzine, A., Rejeb, J., Rebhi, L., Ouedrani, A., Ben Rejeb, N. *et al.* Relationship between leptin G2548A and leptin receptor Q223R gene polymorphisms and obesity and metabolic syndrome risk in Tunisian volunteers. *Genet. Test Mol. Biomarkers* **16**, 726–733 (2012).
65 Lim, U., Ernst, T., Wilkens, L. R., Albright, C. L., Lum-Jones, A., Seifried, A. *et al.* Susceptibility variants for waist size in relation to abdominal, visceral and hepatic adiposity in postmenopausal women. *J. Academy Nutr. Dietetics* **112**, 1048–1055 (2012).
66 Silver, K., Walston, J., Chung, W. K., Yao, F., Parikh, V. V., Andersen, R. *et al.* The Gln223Arg and Lys656Asn polymorphisms in the human leptin receptor do not associate with traits related to obesity. *Diabetes* **46**, 1898–1900 (1997).
67 Gotoda, T., Manning, B. S., Goldstone, A. P., Imrie, H., Evans, A. L., Strosberg, A. D. *et al.* Leptin receptor gene variation and obesity: lack of association in a white British male population. *Hum. Mol. Genet.* **6**, 869–876 (1997).
68 Bender, N., Allemann, N., Marek, D., Vollenweider, P., Waeber, G., Mooser, V. *et al.* Association between variants of the leptin receptor gene (*LEPR*) and overweight: a systematic review and an analysis of the CoLaus study. *PLoS ONE* **6**, e26157 (2011).
69 Heo, M., Leibel, R. L., Fontaine, K. R., Boyer, B. B., Chung, W. K., Koulu, M. *et al.* A meta-analytic investigation of linkage and association of common leptin receptor (*LEPR*) polymorphisms with body mass index and waist circumference. *Int. J. Obes. Relat. Metab. Disord.* **26**, 640–646 (2002).
70 Kolčić, I., Vorko-Jović, A., Salzer, B., Smoljanović, M. m., Kern, J. m. & Vuletić, S. Metabolic syndrome in a metapopulation of Croatian island isolates. *Croat. Med. J.* **47**, 585–592 (2006).
71 Deka, R., Durakovic, Z., Niu, W., Zhang, G., Karns, R., Smolej-Narancic, N. *et al.* Prevalence of metabolic syndrome and related metabolic traits in an island population of the Adriatic. *Ann. Hum. Biol.* **39**, 46–53 (2012).