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Variants of *ENPP1* are associated with childhood and adult obesity and increase the risk of glucose intolerance and type 2 diabetes

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Abstract

We identified a childhood obesity locus on chromosome 6q16.3-q24.2¹ that includes 2.4 Mb common to eight genome scans for Type 2 diabetes (T2D) or obesity¹⁻⁸. Analysis of the *ENPP1* (*PC-1*) gene, a candidate for insulin resistance^{9,10} in 6,147 subjects revealed association between a three allele risk haplotype (K121Q/IVS20 delT-11/A>G +1044 TGA, QdelTG) and childhood obesity (OR=1.69, p=0.0006), and in adults with morbid or moderate obesity (OR= 1.50, p= 0.006, OR= 1.37, p=0.02) and also with T2D (OR=1.56, p=0.00002). The Genotype IBD Sharing Test suggested a contribution of this obesity-associated *ENPP1* risk haplotype to the observed chromosome 6q linkage with childhood obesity. The haplotype confers a higher risk of glucose intolerance and T2D to obese children and to their parents and associates with increased serum levels of soluble *ENPP1* protein in children. Expression of a long *ENPP1* mRNA isoform, which includes the obesity-associated A>G +1044 TGA SNP, was found to be specific for pancreatic islet beta-cells, adipocytes and liver. These findings suggest a primary role for several variants of *ENPP1* in mediating insulin-resistance, in the development of both obesity and type 2 diabetes, suggesting an underlying molecular mechanism common to both widespread afflictions.

Competing interests statement

The authors declare that they have no competing financial interests.

Initially, the phenotypic characteristics of 62 “6q-evidence” families (defined by an individual pedigree Zscore > 1.0 in the 2-LOD drop interval flanked by markers D6S434 and D6S1704) were compared with the remaining 35 families from our previously published genome scan for childhood obesity¹. The “6q-evidence” obese children have a trend towards higher area under the glycemia curve after glucose administration and a significantly lower insulinogenic index (Supplementary Table 1). Compared to none of the other families, 3.1% of the “6q-evidence” obese children are glucose intolerant or diabetic and 13.8% of 6q linked obese children parents have type 2 diabetes mellitus (T2D) compared to 3.2% of parents in other families (p=0.018). Thus, the obesity susceptibility gene(s) on chromosome 6q may be also involved in glucose homeostasis.

The “6q-evidence” 2-LOD drop interval¹ covers 41.4 Mb and includes 166 referenced genes. This was narrowed to a 2.4 Mb interval between markers D6S1656 and D6S270 using overlapping published linkage results on chromosome 6q16.1-q27 with either obesity², insulin secretion^{3,4} or T2D⁵⁻⁸. Within this interval, the best candidate was ectonucleotide pyrophosphatase phosphodiesterase ENPP1 (also known as the Plasma Cell glycoprotein-1 PC-1). ENPP1 is believed to directly inhibit insulin-induced conformational changes of the insulin receptor, thereby affecting its activation and downstream signaling^{9,11}.

The microsatellite marker D6S1656 in intron 1 of the *ENPP1* gene, linked to childhood obesity in our initial genome scan, was analyzed in a second replication set of 68 families with childhood obesity and modest evidence of linkage (MLS=0.83, p=0.04) was observed. Allele 10 of D6S1656 was also significantly under-transmitted to affected children in both the initial and replication sets (44 transmitted vs 69 untransmitted, p uncorrected =0.02).

All coding regions of the *ENPP1* gene were sequenced, plus 1.3 Kb upstream of the ATG start codon and downstream of the TGA stop codon in 48 obese children from “6q-evidence” families and in 24 non-obese adults. Eight single-nucleotide polymorphisms (SNPs) were identified in the upstream sequence, six in intron/exon junctions, four were missense, three were synonymous mutations and twenty were identified in the downstream sequence (Supplementary Table 2). Among these forty-one polymorphisms, twenty-four were present in the public databases and twenty-two of the variants had a minor allele frequency (MAF) higher than 5%. Pairwise LD among the twenty-four most common SNPs was used to select a set of ten “haplotype tagging” SNPs to be typed in the whole set of samples.

Twenty-five intronic or intergenic fragments showing a high degree of homology (>70%) across *Fugu rubripes*, rat and human genomes were also sequenced and a further eleven SNPs of MAF >5% were identified. One SNP, T>G +5954 TGA, that showed a trend for association with childhood obesity (p<0.1) in a test set of 421 obese children and 298 control individuals was added to the set of SNPs analyzed in the whole sample set.

Eleven SNPs were genotyped in 2,430 individuals, made up of 529 unrelated obese children, 696 unrelated morbidly obese adults, and 1,205 lean normoglycaemic adults. Lean adults were used as controls for both sets of cases because they demonstrated a long term resistance against obesity. Associations were observed between severe forms of obesity and six of the SNPs: IVS2 delG +8, K121Q, IVS8 T>G +27, IVS20 delT -11, A>G +1044 TGA and T>G +5954 TGA (0.00008<p<0.03; 1.21<OR<1.37) (see Supplementary Table 3, Table 1). The global p-value, assessed by 10⁵ permutations of the obesity status among individuals, was 0.001. Analysis of the pooled data identified the strongest association with severe forms of obesity of the K121Q SNP (OR=1.37, 95% confidence interval [1.17-1.61], p=0.00008). The odds ratio under a recessive model increased to 3.29 [1.83-5.93] (p=0.00003) with a significant departure from the additive model (p=0.02).

A further 184 multiplex families were then genotyped for K121Q. This consisted of the 97 childhood obesity genome scan families plus 87 nuclear families with adult severe obesity, which showed linkage of serum leptin levels to chromosome 6q24¹². Using the Transmission Disequilibrium Test (TDT) the 121 Q-allele was significantly over-transmitted to obese offspring (transmitted: 76, non-transmitted: 48, $p=0.01$), supporting the case-control result.

To confirm *ENPP1* specificity for these associations, a total of fifty-three SNPs in the chromosome 6q region, spanning 580 kb and including the *ARG1*, *CRSP3*, *ENPP3*, *ENPP1* and *CTGF* genes, were typed in the initial set of 421 obese children and 298 control individuals used above (average density: 1 SNP/10.9 kb). Three distinct regions of linkage disequilibrium (LD) were noted: the first contained *ARG1*, *CRSP3* and *ENPP3*, the second *ENPP1* alone, and the third the *CTGF* gene and 176Kb of non coding region (Fig. 1). Eight SNPs were associated with both childhood obesity and “6q-evidence” childhood obesity ($p<0.05$). Seven of the eight SNPs (K121Q, Celera dbSNP hcV1207989, C>T +164 TGA, Celera dbSNP hcV1207974, A>G +1044 TGA, G>T +1101 TGA, C>T +1157 TGA) mapped within *ENPP1* (Fig. 2), suggesting that the observed association with childhood obesity is due to *ENPP1* SNPs.

A two SNP analysis between the K121Q polymorphism and the 6 other obesity-associated SNPs was used to assess whether these SNPs had an independent effect on the risk of obesity. A likelihood ratio test showed that only IVS20delT-11 and G+1044TGA SNPs significantly modulate the effect of K121Q ($p=0.03$ and $p=0.04$ respectively). It is worth pointing out that for these two SNPs, the model with the best fit was also a recessive one. This may account for the small observed deviation from Hardy-Weinberg Equilibrium (HWE) for these SNPs as genotyping errors have been ruled out by resequencing (data not shown).

To estimate the potential effects of combinations of the three SNPs on the risk of obesity, haplotype analysis was performed using the K121Q, IVS20 delT-11 and A>G +1044 TGA SNP data from the whole set of 2,430 French Caucasian subjects. Eight haplotypes were predicted, five having a MAF >5%. The three allele wild-type haplotype (K121Q/IVS20 delT-11/A>G +1044 TGA, KTA), was less frequent in obese subjects than in controls (60.3% vs 64.0%, $p=0.002$) (Table 2). In contrast, the three allele risk haplotype (K121Q/IVS20 delT-11/A>G +1044 TGA, QdelTG), was strongly associated with severe forms of obesity (11.2% vs 7.5%, OR=1.58, $p=0.00001$, empirical p -value<0.0001 for 10^5 simulations). Interestingly, the risk haplotype effect was of similar magnitude in both morbidly obese adults and childhood obesity (10.8% vs 7.9%, OR=1.50, $p=0.006$, and 11.7% vs 7.1%, OR=1.69, $p=0.0006$, respectively). The association was also supported by TDT analysis in the 184 families ($\chi^2=5.68$, $p=0.01$, Table 2) whereas transmission distortion was excluded in a set of 458 French Caucasian trios with unaffected children ($\chi^2=0.53$, $p=0.46$)¹³. Finally, the risk haplotype was associated with obesity (10.3% vs 7.9%, OR=1.37, $p=0.02$) in an additional set of 717 adult subjects with a less severe form of obesity (BMI between 30 and 40 kg/m²).

The impact of the *ENPP1* risk haplotype on the linkage with childhood obesity observed in the genome scan was identified in several ways. Initially, a higher frequency of the risk haplotype in individuals from “6q-evidence” families (16.2% vs 7.1%, OR=2.37, $p=0.004$) compared to other families (12.2% vs 7.1%, OR=1.65, $p=0.16$) was detected. After removing 15 affected sib-pairs sharing the risk haplotype (total sib-pairs = 135), the multipoint MLS LOD score dropped from 4.06 to 1.6 at marker D6S287, and a new maximal score of 2.63 appeared 16-Mb centromeric to the original linkage peak, at marker D6S301. The Genotype IBD Sharing Test (GIST)¹⁴ also suggested a trend for a possible effect of the haplotype under an additive model ($p=0.07$) which became significant for a recessive model ($p=0.03$).

At least three *ENPP1* SNPs are involved in the association with obesity. The Q121 variant is believed to inhibit insulin signalling¹⁵ more effectively than the wild-type version, but the

functional effects of the risk haplotype are unknown. The ENPP1 protein has a proteolytic cleavage site, is cleaved at the surface of cells, and is known to be present in the circulation¹⁶. Thus, the protein serum level represents a good estimation of its tissue expression¹⁶. Serum ENPP1 protein levels measured in 279 children encompassing a wide weight range showed a positive correlation with the Z score of BMI (Pearson correlation coefficient=0.1, $p=0.05$, Supplementary Figure 1). Eighty-nine lean children were then selected (mean BMI, 18.4 ± 2.5 kg/m²; mean age, 13.3 ± 2.6 y; $n=50/39$ girl/boy), to fix the confounder BMI, and analyzed for the effect of the three SNPs on ENPP1 levels. The presence of at least one copy of Q121, IVS20delT-11 and G+1044TGA alleles was associated with a highly significant increase of ENPP1 levels (28.6 ng/ml vs 24.1 ng/ml, $p=0.008$) (Fig. 3), suggesting that the obesity-associated haplotype not only impairs insulin binding but also enhances ENPP1 levels of expression.

The contribution of the risk haplotype on the variation of obesity-related phenotypes was then assessed in 474 obese children where data were available. Obese children with the obesity-associated risk haplotype showed a 0.17 mmol/l increase in fasting glycemia ($p=0.002$) with a higher prevalence of glucose intolerance/T2D (OR=3.43, $p=0.02$). Parents carrying the risk haplotype had a 2.35 fold increased risk to develop T2D ($p=0.005$). This risk was higher in the subset of obese parents (OR=3.26, $p=0.0005$) with no increase in the risk of T2D observed in non obese parents carrying the risk haplotype (OR=0.86, $p=0.9$). An additional non-overlapping cohort of 752 unrelated T2D French Caucasian subjects with familial history of the disease was compared to the previously used 556 middle aged non-obese normoglycemic subjects (average age: 55 ± 6 years). The T2D group had a significant excess of the risk haplotype (10.7% vs 7.1%, OR=1.44, $p=0.005$) further supporting a potential effect of *ENPP1* SNPs on glucose homeostasis in French Caucasians. This finding was replicated using 1261 unrelated Austrian subjects consisting of 503 T2D subjects and 758 non obese normoglycemic subjects (9.8% vs 6.3%, OR=1.68, $p=0.001$). The Mantel-Haenszel adjusted odds ratio, under a fixed effects model, was used in the pooled cohorts of 2569 European subjects, and strengthened the association of the risk haplotype with T2D (10.4% vs 6.6%, combined OR=1.56, $p=0.00002$). In summary, these findings indicate that both obesity and T2D, especially in obese subjects, are associated with *ENPP1* genetic variability and specifically one three-allele risk haplotype. This provides genetic evidence for the recently described link between obesity in childhood and the high risk for T2D in the teens or early adulthood¹⁷, providing the first common molecular mechanism for this deleterious association.

RT-PCR was performed on cDNAs from a wide range of human tissues including brain, muscle, liver, adipocyte (subcutaneous and omental), and purified pancreatic islet beta-cells. Taking into account that five ENPP1 isoforms are known, (<http://www.ncbi.nih.gov/IEB/Research/Acembly/av.cgi?db=human&l=ENPP1>), primers were designed to amplify the region between exons 7 and 12, common to at least 3 transcripts, and ubiquitous expression was found (Supplementary Figure 2). Primers were then designed to specific amplify the long mRNA isoform, (larger 3'UTR with 1170 bases downstream of the TGA stop codon) that includes the obesity-associated SNP A>G +1044 TGA. The long form was found to be only expressed in pancreatic beta-cells, adipocytes and liver, three key tissues for glucose homeostasis (Supplementary Figure 2).

The contribution of the obesity-associated *ENPP1* risk haplotype to our observed linkage was assessed by several methods but only a moderate excess of transmission of this haplotype to affected offspring was found. This suggests that the risk haplotype is contributory but not sufficient to explain the linkage with childhood obesity. Additional SNPs in the non-coding regions of the *ENPP1* locus may account for part of the observed linkage, including SNP T>G +5954 TGA, located in a highly conserved region. This variation predicts loss of binding to

the Insulin Promoter Factor IPF1. Alternatively, more than one gene may explain the linkage with obesity and T2D on 6q. Recent data supports a contribution of *ENPP1* SNPs¹⁸ to linkage for insulin fasting levels in Mexican-Americans³.

Only the *ENPP1* K121Q exon 4 missense mutation has been suggested to associate with insulin resistance or T2D in limited studies¹⁹. The 3'UTR (A>G +1044 TGA) SNP belongs to an isoform specifically expressed in three highly insulin-responsive human tissues (pancreatic islet beta-cell, adipocyte and liver). Mice given an adenovirus expression construct overexpressing this gene in hepatocytes show insulin resistance and glucose intolerance¹⁰. Although the exonic 121Q amino acid substitution directly inhibits insulin receptor by a non enzymatic mechanism¹⁵, the other non-coding SNPs may have their effect by modifying gene expression, protein production or splicing. This hypothesis is favoured by the increased serum protein levels in children carrying the *ENPP1* obesity risk haplotype and by the rise of *ENPP1* levels with adiposity.

Higher protein expression may mimic the effects of insulin receptor inactivation in the brain where insulin has potent anorectic actions, or in the skeletal muscle, both leading to an increased fat mass^{20,21}. Obese children carrying the *ENPP1* risk haplotype often have glucose intolerance and a family history of T2D. This suggests that the exaggerated insulin resistance conferred by inherited increased *ENPP1* expression in the context of a Westernized obesogenic environment, may contribute to excessive fat accumulation. The *ENPP1* Q121 allele was recently associated with increased BMI in the UK general population²². Data presented here supports the view of a causative effect of primary insulin resistance on childhood obesity. According to this hypothesis, insulin resistance-induced fasting hyperinsulinemia was shown to be a strong predictor for the subsequent development of obesity in children of various ethnic groups²³.

In conclusion, this study strongly supports a genetic link between *ENPP1* gene variants and chromosome 6q-linked childhood polygenic obesity and also with adult obesity and T2D. This provides an insight into the molecular basis for the physiologic association between insulin resistance and obesity, and presents a new perspective for prevention and treatment of these conditions.

Methods

Subjects

Phenotypic characteristics are summarized in Supplementary Table 4. Five hundred and twenty nine unrelated obese children were studied. We collected 336 pedigrees with at least one obese child. One hundred and six additional obese children were recruited in Toulouse, and 87 in Paris. Children with a BMI greater than the 97th percentile of BMI for age and sex were diagnosed as obese. We used a set of 696 unrelated morbidly obese (BMI ≥ 40 kg/m²) and 717 unrelated moderately obese (BMI between 30 and 40 kg/m²) adult patients. We also used for T2D 87 pedigrees with adult obesity. The 752 T2D subjects were recruited at the Sud Francilien Hospital or at the CNRS-Lille. The first set of 556 control subjects was obtained from the SUVIMAX population²⁴. The second set of 649 control individuals was recruited at the CNRS-Lille and through the "Fleurbaix-Laventie Ville Santé" study. The 1,261 Austrian subjects were previously described²⁵. In order to justify the use of non-age matched controls we genotyped the K121Q, IVS20 delT -11 and A>G +1044 TGA polymorphisms in 198 control children from²⁶ and found very concordant frequencies for the risk haplotype (7.8%) in comparison with control adults. The 458 control trios (2 parents and one lean child) were issued from the "Fleurbaix-Laventie Ville Santé" cohort²⁶. The genetic study was approved by Ethical Committees of Hotel Dieu in Paris and CHRU in Lille. The Z score of BMI was obtained according to Cole's method. Insulinogenic index was calculated according to Seltzer *et al.*²⁷.

Linkage analysis

In sixty-eight nuclear families, comprising 306 individuals, five polymorphic markers (D6S1720, D6S434, D6S287, D6S1656, D6S292) covering the linkage interval of the initial genome-wide study¹ were used for genotyping. Two-point and multipoint analyses were performed using the MLS test implemented in the GeneHunter software.

Mutation screening

The twenty-five exons and UTRs (upstream 5' UTR and downstream 3' UTR) were screened using DHPLC (Transgenomic, San Jose, Calif., USA) in 48 unrelated obese children randomly selected among families contributing to the linkage at the chromosome 6q16-q24 locus and in 24 unrelated non-obese normoglycemic control subjects selected from French pedigrees. Variant profiles were sequenced using an automated ABI Prism 3700 DNA sequencer in combination with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, Calif., USA).

Genotyping

Genotyping of microsatellites was carried out using a fluorescence-based semi-automated technique on automated DNA sequencing machines (ABI 377, PE ABI). Primers were synthesized by MWG Biotech. SNPs were genotyped with the LightCycler™, LightTyper™ (Roche Diagnostics, Basel, Switzerland), with the TaqMan™ (Applied Biosystems, Foster City, Calif. USA) or by direct sequencing. Probes for LightCycler™ and LightTyper™ were synthesized by TIB Molbiol™ Syntheselabor Germany. For coverage of the 580 kb region, SNPs with an allele frequency > 10% in Caucasians were selected using the Applied Biosystem SNP Viewer II software. In order to avoid SNP genotyping errors 10% of DNA samples were systematically re-genotyped for further verification. We found concordance rates of 100% for all SNPs.

Statistical analyses

Comparison of allele frequencies between cases and controls was achieved using the χ^2 test and the *p* value was empirically computed with the program CLUMP. Haplotype frequencies were determined and were compared between groups with the UNPHASED software <http://www.mrc-bsu.cam.ac.uk/personal/frank/>. The effect of haplotypes on qualitative or quantitative trait variation was evaluated using the sub-programs cocaphase and qt-phase of UNPHASED software. Independence of association was tested with the software THESIAS²⁸. THESIAS also implements an EM algorithm and allows for likelihood testing of models of haplotype effect in a linear framework. We used this program to test whether the effect of each SNP, on the obesity status, was independent from the effect of K121Q alone. TDT analyses on SNPs and haplotypes were performed by the TDT method implemented in the UNPHASED software. Because of low LD between the three SNPs of the risk haplotype, we only used the unambiguous haplotypes to obtain a true TDT robust to population stratification. In order to evaluate the effect of the risk haplotype on linkage, we used the Genotype IBD Sharing Test (GIST) procedure¹⁴.

Linkage disequilibrium analysis

LD among twenty-four identified *ENPP1* SNPs and among fifty-three SNPs in the 580 kb *ENPP1* region was investigated. Pairwise delta (correlation coefficient between SNPs) was estimated from genotypes and the results were visualized by the GOLD program (<http://www.sph.umich.edu/csg/abecasis/GOLD/>).

ENPP1 mRNA expression

Human cDNAs from MTC Panel (BD Biosciences Contech), subcutaneous and omental adipocytes (provided by G Fruhbeck, university of Navarra Pamplona Spain) and FACS purified pancreatic beta cells (provided by the Human Pancreatic Cell Core Facility, University Hospital, Lille, France) were used for mRNA expression analysis. The beta cell purity was confirmed by immunochemistry (98% insulin positive cells) and PCR (absence of amplification with chymotrypsin primers, specific for exocrine cells). PCR was performed in a 25 µl mixture containing 10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, 10 mM of each dNTPs, 2.5 U of *Taq* polymerase (Promega), 30 mM of both forward primer and reverse primer, and 3 µl of single-strand cDNA. The mixtures were heated at 95°C for 2 min and subjected to 45-cycle amplification at 95°C for 30 s, 68°C for 2 min, and 72°C for 2 min, and then 10 min at 72°C. PCR products were separated on 2% (w/v) agarose gel and visualized using ethidium bromide and UV transillumination.

ENPP1 protein serum assays

ENPP1 serum level was measured using the ELISA described by Rutsch *et al.*¹⁶.

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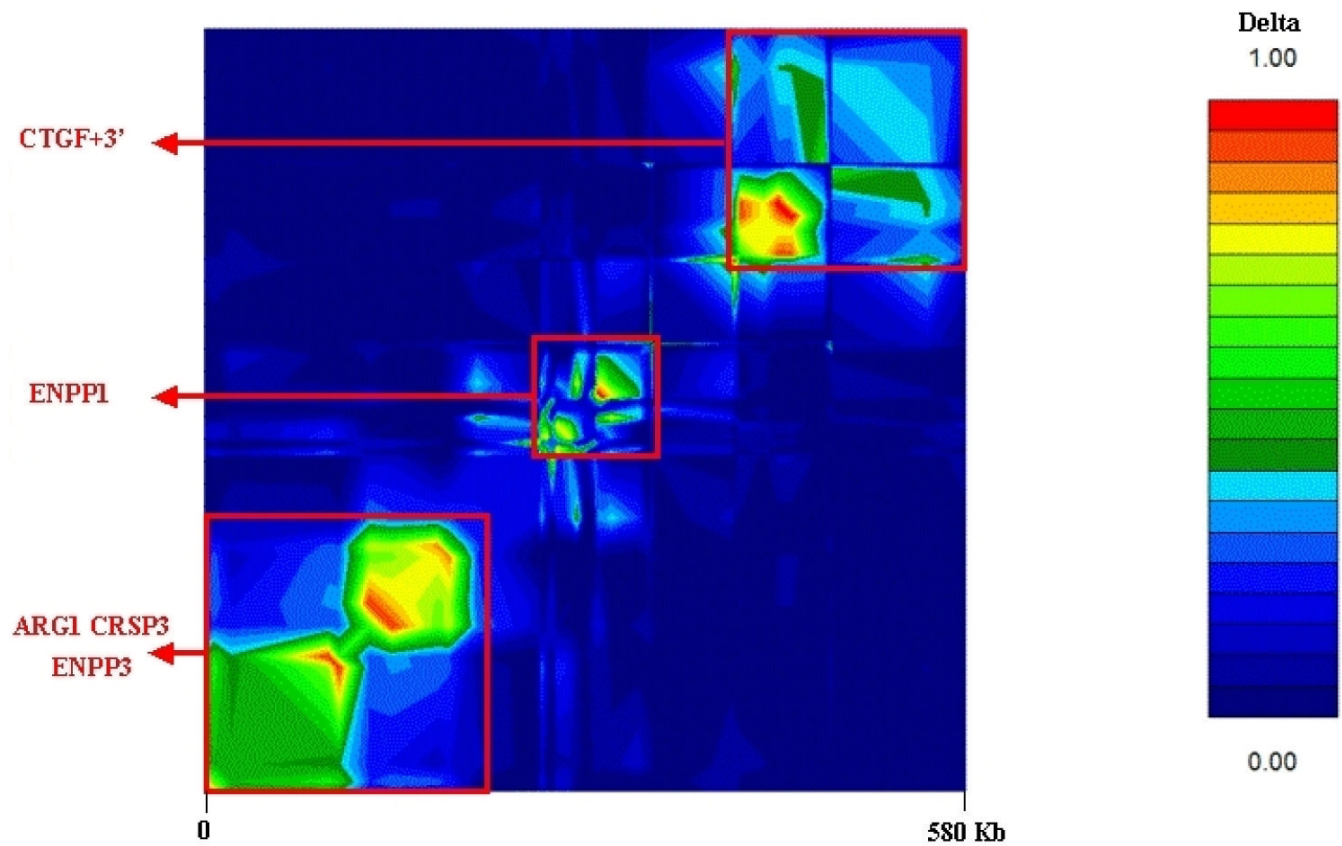


Figure 1. Pairwise LD between fifty-three SNPs in a 580 kb region including the *ENPP1* gene in 421 obese children and 298 control individuals. Regions of high and low LD (delta) are presented by red and blue shading, respectively. The graph is to the physical map scale.

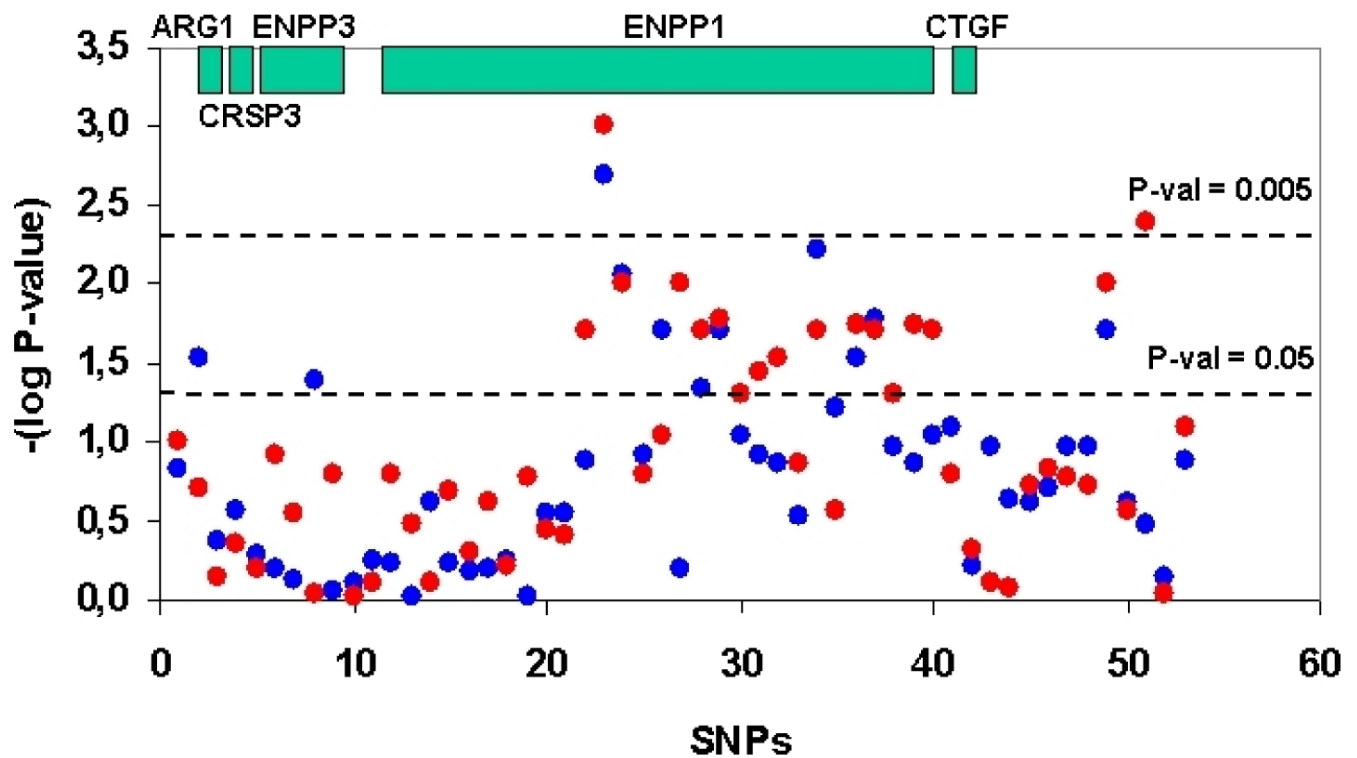


Figure 2.

Allelic association of fifty-three SNPs in the 580 kb region including the *ARG1*, *CRSP3*, *ENPP3*, *ENPP1* and *CTGF* genes. Blue circles correspond to the $-\log_{10}(p\text{-value})$ for the comparison of allelic distribution between 421 obese children and 298 control individuals. Red circles correspond to the $-\log_{10}(p\text{-value})$ for the comparison of allelic distribution between 62 "6q-evidence" families obese children and 298 control individuals. The figure is not to scale according to the physical map location; indeed the SNPs are equidistant to allow easy visualization of the association test results.

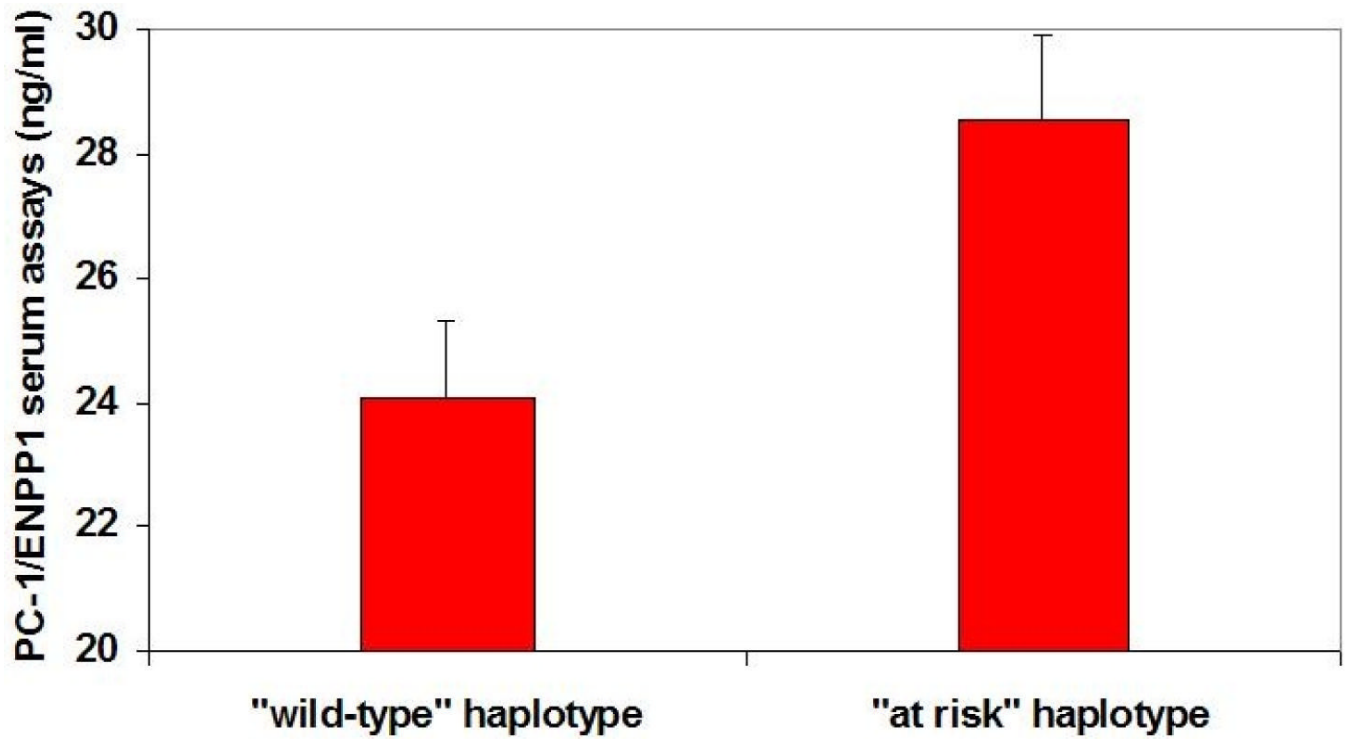


Figure 3.
ENPP1 serum level in 89 lean children according to the presence of the risk haplotype.

Table 1

Case-control analysis of *ENPPI*

SNP	Location	Allele (1/2)	Allele 2 frequency		Genotype 11+12 versus 22		
			Affected individuals	Controls	OR (95% c.i.)	χ^2	P
K121Q	Exon 4	A/C	0.18	0.13	3.29 (1.83-5.93)	17.5	0.00003
IVS20delT-11	Intron 20	T/delT	0.26	0.23	1.89 (1.34-2.67)	13.4	0.0002
A>G+1044 TGA	3'UTR	A/G	0.31	0.27	1.76 (1.31-2.37)	14.1	0.0002

SNPs with P<0.0005 in allele frequency comparison test are shown. c.i., confidence interval; OR, odds ratio.

Haplotype analysis of 1225 morbidly obese/obese children and 1205 control subjects
Haplotypes comprising three polymorphisms (K121Q, IVS20 delT-11, A>G+1044 TGA) are shown. TDT was performed for each haplotype in 97 pedigrees with childhood obesity and in 87 pedigrees with adult obesity.

Haplotypes		Case/control Test			TDT in 184 obesity pedigrees	
K121Q	IVS20delT-11	A>G+1044TGA	Non obese	Obese	Chi-square	p-value
K	T	A	64.0	60.3	8.99	0.003
		G	7.0	7.6	0.51	0.47
	DelT	A	3.5	2.7	1.26	0.26
Q	T	G	11.7	11.9	0.18	0.67
		A	5.5	4.8	0.15	0.70
	DelT	G	0.6	0.6	0.19	0.66
	DelT	A	0.2	0.9	5.23	0.02
		G	7.5	11.2	18.78	0.00001
					5.68	0.01