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ORIGINAL ARTICLE

Possible role for *ENPP1* polymorphism in obesity but not for *INSIG2* and *PLIN* variants

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Abstract Previous studies have suggested that *ENPP1*, *INSIG2*, and *PLIN* may be linked with a higher risk for obesity or with increased phenotypic measures of obesity. We selected polymorphisms in these candidate genes based on their prior associations with obesity risk or obesity parameters. K121Q (rs1044498) in *ENPP1*, rs7566605 in *INSIG2*, and rs894160 in *PLIN* were genotyped by Taqman assays in a Belgian sample of 1,078 obese subjects (body mass index (BMI) > 30 kg/m^2) and 323 lean controls ($18.5 <$ BMI < 25 kg/m^2). BMI, waist circumference, and waist-to-hip ratio (WHR) were assessed by standard methods while a computerized tomography-scan was used to measure visceral (VFA), subcutaneous (SFA), and total (TFA) abdominal fat areas. Presence of the rare allele was not significantly different between cases and controls for the three variants that were tested, while only WHR was associated with *ENPP1* in obese subjects. Our data thus indicate that K121Q, rs7566605, and rs894160 are not major contributing factors for obesity.

Keywords Association studies · ENPP1 · INSIG2 · Perilipin · Obesity

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Introduction

Obesity is a chronic illness of excess body fat that can manifest as a monogenic disease resulting from a single gene mutation, or as a complex disorder when several gene variants, as well as environmental factors, contribute to the phenotype. Complex obesity is most common, having risen rapidly to epidemic proportions over the last two decades while the WHO predicts that 700 million adults will be obese by 2015 [1]. Genome-wide scans as well as screenings of candidate regions have enabled identification of single nucleotide polymorphisms (SNPs) in various genes, which increase the risk of becoming overweight and obese. *ENPP1*, *INSIG2*, and *PLIN* are three such loci that have recently been linked to adiposity.

The *ENPP1* gene encodes ectonucleotide pyrophosphatase phosphodiesterase 1, which is a transmembrane glycoprotein that inhibits tyrosine kinase activity of the insulin receptor, thereby decreasing insulin signaling in cells [2]. *ENPP1* is an attractive candidate gene for obesity since it lies on chromosome 6q in a region where at least two independent studies found a linkage peak to obesity [3, 4]. Consequently, several groups have attempted to confirm through population-based association studies that variants in *ENPP1* are responsible for obesity-related phenotypes and these analyses have produced both positive and negative results [3–16].

INSIG proteins bind sterol regulatory element binding protein (SREBP) cleavage-activating protein (SCAP), thus obstructing the proteolytic processing of the SREBPs into transcription factors [17]. While INSIG1 is primarily involved in cholesterol homeostasis, INSIG2 has a role in human adipocyte metabolism seeing that adipocyte differentiation is linked to an increased expression of *INSIG2* [17]. In a recent genome-wide association analysis for

elevated body mass index (BMI), a polymorphic variant upstream of the *INSIG2* gene (insulin-induced gene 2) on chromosome 2 was identified for the first time [18], although mouse and human linkage studies had already identified *INSIG2* as an obesity QTL [19, 20]. Despite successful replication by Herbert et al. of their finding in four out of five samples from different populations [18], subsequent studies have yielded only negative results [21–35].

On the long arm of chromosome 15 resides the *PLIN* gene for perilipin, which is the major member of the perilipin phosphoprotein family. Perilipin is essential for the mobilization of triglycerides because it is responsible for coordinating the transfer of surplus energy as triacylglycerols into lipid droplets in adipocytes [36]. The fact that *PLIN* knockout mice are lean and resistant to diet-induced obesity [37] as well as the finding of polymorphisms that are linked to BMI and obesity risk [38, 39] suggest that *PLIN* is a good candidate gene for obesity. Nevertheless, for *PLIN* also results of replication studies have been inconsistent [40–44].

In summary, causative links to obesity have previously been established with, among others, the K121Q variant (rs1044498) in the *ENPP1* gene, the rs7566605 *INSIG2* promoter polymorphism, and SNP 11482G>A (rs894160) in the gene for perilipin (*PLIN*) and these analyses have been repeated in several cohorts. Nevertheless, data are still conflicting and we therefore genotyped these variants in a Belgian case–control sample to test whether these polymorphisms contribute to the development of common obesity.

Results

Characteristics of the study population are shown in Table 1. A total of 1,078 obese patients and 323 lean control individuals were genotyped for rs1044498 (*ENPP1*), rs7566605 (*INSIG2*), and rs894160 (*PLIN*) (Table 2). The minor allele frequency (MAF) of these SNPs in our control population was 0.11, 0.31, and 0.27, respectively. Both the obese and the control group were in Hardy–Weinberg equilibrium (HWE) for all SNPs analyzed ($P > 0.01$; not shown). Chi-square analysis indicated that, for the three SNPs that were analyzed, the distribution of genotypes was similar between cases and controls (Table 2). In addition, the *P*-values of the calculated odds ratios (ORs) were not significant for any of the models tested, thus implying that neither of the three variants contributes to the risk for obesity (Table 2). We analyzed the obesity parameters waist and waist-to-hip ratio (WHR) in the obese population for association with the tested SNPs as well as total (TFA), visceral (VFA), and subcutaneous (SFA) abdominal fats that were based on computerized tomography (CT)-scan measurements and for which we calculated a pairwise Pearson correlation with BMI of 0.86, 0.48, and 0.75 ($P < 0.01$), respectively. The association analysis revealed a significant effect of *ENPP1* genotype on WHR (dominant model; $P = 0.004$ unadjusted; $P = 0.005$ adjusted for age and BMI) (Table 3). Rs7566605 in *INSIG2* and rs894160 in *PLIN* were not associated with any of the obesity parameters that were studied (Table 3).

Table 1 Characteristics of the study population

	Lean ($n = 323$)	Obese ($n = 1,078$)	*P*
Males/females	100/223	469/609	
Age (years)	35.9 ± 0.4	42.6 ± 0.4	<0.001
Weight (kg)	63.9 ± 0.6	110.7 ± 0.7	<0.001
Height (cm)	168.1 ± 0.8	170.0 ± 0.3	0.02
BMI (kg/m^2)	22.1 ± 0.1	38.2 ± 0.2	<0.001
Waist (cm)	76.8 ± 0.6	116.2 ± 0.4	<0.001
WHR	0.77 ± 0.01	1.00 ± 0.01	<0.001
TFA (cm^2)	277 ± 15	755 ± 5	<0.001
VFA (cm^2)	56 ± 3	188 ± 3	<0.001
SFA (cm^2)	221 ± 13	569 ± 4	<0.001

Data are shown as mean ± standard error of the mean

P Result of *T*-test, *WHR* waist-to-hip ratio, *TFA* total fat area, *VFA* visceral fat area, *SFA* subcutaneous fat area as measured by CT-scan

Discussion

We investigated three SNPs in different candidate genes that had previously been found to contribute to obesity but we could not confirm the associations reported in some studies between any of the variants and the risk for obesity. Moreover, we were able, for the first time, to exclude a relationship between the SNPs analyzed and fat mass as measured by CT-scan. Though the original reports of the three candidate genes could link genetic variants with the odds of developing an obesity phenotype, follow-up studies have yielded inconsistent results.

In our study, *ENPP1* rs1044498 genotype cannot be linked to obesity risk but nevertheless, this polymorphism is significantly associated with WHR in obese subjects in a dominant model and remains significant after correcting for multiple testing. The first study of *ENPP1* as a candidate gene for obesity was conducted in a large cohort with sufficient power to detect relatively weak associations [3]. Six *ENPP1* SNPs were found to be associated with morbid obesity and a three-SNP haplotype was linked to obesity as well. Since the K121Q variant showed the strongest

Table 2 Case/control association study of variants in candidate genes for obesity

	ENPP1		*INSIG2*			*PLIN*		
	rs1044498		rs7566605			rs894160		
	Lean	Obese		Lean	Obese		Lean	Obese
AA	257	813	GG	154	497	GG	173	561
AC	60	236	GC	136	476	GA	123	430
CC	6	29	CC	33	105	AA	27	87
MAF	0.11	0.14	MAF	0.31	0.32	MAF	0.27	0.28
χ^2 (P)[a]	2.54 (0.28)		0.43 (0.81)			0.34 (0.84)		
Additive OR (95% CI)[b]	1.18 (0.90–1.55)		1.02 (0.84–1.24)			1.03 (0.84–1.26)		
P^{c}_{additive}	0.23		0.84			0.76		
Dominant OR (95% CI)[d]	1.20 (0.87–1.64)		1.04 (0.81–1.35)			1.04 (0.80–1.34)		
P^{e}_{dominant}	0.27		0.75			0.78		
Recessive OR (95% CI)[f]	1.41 (0.57–3.50)		0.98 (0.64–1.50)			1.04 (0.65–1.66)		
$P^{g}_{\text{recessive}}$	0.46		0.92			0.87		

Genotype counts in lean control and obese subjects.

[a] Genotype distributions are compared by chi-square analysis with 2 degrees of freedom.

[b,d,f] Odds ratios

[c,e,g] *P* values are adjusted for age and gender and estimated by logistic regression for an additive, dominant, and recessive model, respectively

MAF Minor allele frequency, *OR* odds ratio, 95% *CI* 95% confidence interval

Table 3 Results of association analysis for five obesity parameters in three candidate genes in obese subjects

	Additive model		Dominant model		Recessive model	
Genotypes	AA versus AC versus CC		AA versus AC + CC		AA + AC versus CC	
ENPP1	*P*	*P**	*P*	*P**	*P*	*P**
Waist	0.365	0.092	0.158	0.082	0.788	0.456
WHR	0.018	0.018	**0.004**	**0.005**	0.436	0.423
TFA	0.623	0.545	0.331	0.411	0.744	0.347
VFA	0.501	0.637	0.613	0.628	0.385	0.520
SFA	0.159	0.176	0.077	0.069	0.843	0.884
Genotypes	GG versus GC versus CC		GG versus GC + CC		GG + GC versus CC	
INSIG2	*P*	*P**	*P*	*P**	*P*	*P**
Waist	0.668	0.821	0.480	0.803	0.751	0.634
WHR	0.724	0.923	0.497	0.774	0.540	0.725
TFA	0.129	0.443	0.082	0.231	0.648	0.955
VFA	0.810	0.188	0.790	0.495	0.627	0.157
SFA	0.376	0.546	0.256	0.421	0.664	0.338
Genotypes	GG versus GA versus AA		GG versus GA + AA		GG + GA versus AA	
PLIN	*P*	*P**	*P*	*P**	*P*	*P**
Waist	0.623	0.913	0.440	0.972	0.748	0.677
WHR	0.972	0.993	0.811	0.910	0.960	0.939
TFA	0.566	0.922	0.290	0.805	0.646	0.703
VFA	0.710	0.962	0.847	0.995	0.409	0.791
SFA	0.255	0.611	0.099	0.365	0.656	0.502

Three genetic models were tested

Values that are bold indicate associations that are significant after controlling the FDR [54]

P Result of test on unadjusted parameters, *P** result of test on studentized residuals corrected for age and BMI, *WHR* waist-to-hip ratio, *TFA* total fat area, *VFA* visceral fat area, *SFA* subcutaneous fat area as measured by CT-scan

association when analyzed separately, it has subsequently been genotyped by others in several cohorts. The results of these studies have failed, however, to confirm the initial findings by Meyre and coworkers [7, 10, 11, 45] since most of these studies found no association of K121Q with obesity or BMI. In addition, in the few studies that did find a significant effect on BMI, the Q121 allele was associated with higher BMI in some reports [12, 14] and with lower BMI in others [6, 13]. Furthermore, two large studies in the UK and the US, both with adequate replication power, could not affirm the correlation between K121Q and obesity [8, 9]. On the contrary, the UK study was able to exclude an effect of K121Q on obesity with >95% confidence [8]. Also, a study by Stolerman et al. who genotyped 39 *ENPP1* tagSNPs in participants from the Framingham Heart Study (FHS), found no association of K121Q with BMI or waist circumference, although a higher BMI strengthened the association of K121Q with hyperglycemia and insulin resistance [5]. This is in agreement with a recent analysis in Mexican Americans of 106 SNPs within *ENPP1*, which also found only modest evidence of an association of *ENPP1* variants with the metabolic syndrome but not with obesity [4]. Finally, a case–control study in children from Naples again did not observe a significant association between K121Q and obesity, whereas 121Q allele carriers had higher insulin and HOMA values than subjects with the wildtype genotype [16]. Thus, in view of the above, it seems reasonable to suggest that K121Q may have an effect on parameters of the metabolic syndrome while the reported associations with obesity are probably chance findings.

Our analysis of variant rs7566605—upstream of *INSIG2*—did not reveal any meaningful correlation with obesity risk nor with quantitative traits of obesity (Table 3). The *INSIG2* gene was originally linked to obesity by Herbert et al. who found that in a whole-genome scan of subjects from the FHS offspring cohort only SNP rs7566605 reached significance for association with BMI [18]. They subsequently obtained the same result in four other cohorts while in a fifth sample, the confidence interval for the OR for obesity was not significant. It is intriguing, then, that most studies initiated afterward in different population samples found no evidence to support the claim by Herbert et al. Indeed, rs7566605 was significantly associated with BMI in a Chinese subpopulation and with morbid obesity in a Japanese population, while 10 other reports were negative [23–31, 33]. Lyon et al. [46], in a large multi-ethnic study, did find an association in five cohorts but could not confirm the association in three other cohorts, while the combined analysis was also significant. They suggest a minor effect of rs7566605, which may be masked by confounders such as small sample size or population stratification. In addition, they provide a reasonable explanation for the reported discrepancies in that they propose that the effect of rs7566605 on BMI may be heterogeneous in different populations. Finally, detailed functional studies conducted by Krapivner et al. [17] indicate that INSIG2 is indeed involved in adipocyte metabolism and weight regulation but they suggest that the −102G/A polymorphism, and not rs7566605, is the causative SNP which underlies the association of *INSIG2* with BMI. Summing up the results for rs7566605, this polymorphism most probably has a small effect on obesity risk but other SNPs within or close to the gene's coding region (e.g., −102G/A) may be better candidates for a causative variant.

Finally, examination of rs894160 in the *PLIN* gene in our study sample also did not yield any probability values below the corrected significance level (Table 3). The first report to link the perilipin locus to the risk for obesity was an analysis of six *PLIN* SNPs in a randomly selected population from Eastern Spain [38]. In this study, rs894160 was one of two SNPs associated with lower measures of body weight, WHR, plasma glucose as well as triglycerides and the variant allele also reduced obesity risk in white women [38]. Soon after, another study of four SNPs in *PLIN* revealed a significant association with body adiposity in white subjects for two variants, but not with rs894160 [39]. This lack of support for the original findings can likely be attributed to the differences in selection criteria since the latter study consisted of overweight and obese individuals who were following a lifestyle intervention program. Next, Qi et al. [44] studied five *PLIN* SNPs and their haplotypes in a multiethnic Asian population and found a significantly increased obesity risk for the rare allele of rs894160 but only in the Malay sample. However, a study by Meirhaeghe et al. [42] could not confirm the association in a random study sample, which included French obese subjects and type 2 diabetes patients. While seven SNPs were tested, only two of them were frequent enough to be analyzed, one of which was rs894160. Finally, a study of perilipin gene variation in two German cohorts also did not find any significant associations between three *PLIN* variants and obesity, although rs894160 was not included in this analysis [40]. Most recently, Deram et al. found a significant association between rs894160 and an increased risk for the metabolic syndrome at baseline in a population of obese children and adolescents from Brazil; however, once again rs894160 could not be linked to obesity [41]. Considering the abovementioned *PLIN* analyses, it is difficult to substantiate a putative role for rs894160 in obesity. In addition, the associations between other *PLIN* variants and obesity were also inconsistent and adequately powered studies in large homogeneous populations are needed to resolve the ambiguity.

It should be noted that some of the past studies reviewed here consisted of randomly selected individuals while others included persons with diabetes or obese and overweight

individuals and these dissimilarities may reasonably explain the lack of replication of the associations of *ENPP1*, *INSIG2*, and *PLIN* and obesity. Additionally, populations with a distinct genetic background, like most of the above-mentioned studies, are difficult to compare and failure to confirm an association in a population of different ancestry is not unusual [47]. Finally, gene–gene interactions (e.g., [48]), publication bias and population stratification are other plausible reasons for the lack of correspondence between association studies [49]. Our nonreplication of associations reported in the past between SNPs in the selected candidate genes and obesity is unlikely to have been caused by population stratification. Since all our subjects are Belgians with Belgian parents, the likelihood of population admixture is small. Likewise, analysis of 10 intergenic SNPs spread across the genome in a small subset of our cases and controls matched on age and gender did not show any indication of population stratification [50].

Our study has some strengths and limitations. We explored a homogeneous sample of well-characterized cases and controls, which increases sensitivity for detecting associations. In addition, we concentrated on SNPs that putatively have a functional effect as two out of three SNPs that we analyzed, i.e., rs1044498 (*ENPP1*) and rs894160 (*PLIN*) are found within the coding region of the candidate gene. Furthermore, we investigated, for the first time, putative associations of SNPs in *ENPP1*, *INSIG2*, and *PLIN* with CT-scan data as quantitative traits. However, since only one SNP was analyzed in each of the three genes, we cannot exclude with certainty that variants in other regions of the candidate genes are associated with obesity. A comprehensive association study using tagSNPs to capture the genetic information of the entire candidate gene could possibly yield associations that are missed otherwise. Finally, we realize that extension of our study population, as well as a better case-to-control ratio would also improve the statistical power of our analysis. Nevertheless, although we cannot exclude false-negative results, it should be pointed out that we have been able to confirm the association of two SNPs in the *FTO* gene with obesity in the same population sample [51], which validates the results of our present analysis.

In conclusion, we found no evidence of an association between the studied SNPs in *ENPP1*, *INSIG2*, and *PLIN* and the risk of obesity whereas the result for *ENPP1* warrants further study in a larger population sample.

Materials and methods

Subjects

All subjects included in this study were Belgian Caucasians older than 20 years, with Belgian Caucasian parents and at enrollment none were involved in an ongoing weight management program. A total of 1,078 obese patients with a BMI of at least 30 kg/m^2 were recruited in chronological order from the weight management clinic at the Antwerp University Hospital. A total of 323 lean control individuals had a BMI between 18.5 and 25 kg/m^2, and were volunteers from among the staff at the university hospital and the Department of Medical Genetics. The study protocol was approved by the ethics committee of the Antwerp University Hospital and all study persons had given their written informed consent before participation.

Anthropometric measurements

Height was measured to the nearest 0.5 cm, body weight was determined with a digital scale to the nearest 0.1 kg. BMI was calculated as weight (in kg) over height (in m) squared. Waist circumference was measured at mid-level between the lower rib margin and the iliac crest, and hip circumference at the level of the trochanter major and the WHR was calculated. VFA, SFA, and TFA areas were determined with a CT-scan at the L4–L5 level, according to the technique described by Van der Kooy et al.

Genotyping

Genomic DNA was extracted from whole blood by a method adapted from Miller et al. [52] and 5 ng genomic DNA per well was dispensed in 384-well plates with a Tecan Genesis RSP100 liquid handling robot. Prior power calculations were carried out with the CaTS power calculator [53]. We have 84% prior power to detect associations of SNPs with a MAF $\geq$ 0.20 and a genotype relative risk of 1.3. The selected SNPs, rs1044498 (K121Q in exon 4 of ENPP1); rs7566605 (10 kb upstream of the *INSIG2* start codon); and rs894160 (the 11482G>A variant in intron 6 of PLIN) were obtained as pre-designed TaqMan SNP genotyping assays (ABI, Foster City, USA) and analyzed in 5-μl reactions containing 5 ng genomic DNA on a LightCycler 480 Real-Time PCR System (Roche; Penzberg, Germany). We used standard running conditions as recommended by the manufacturer. Blank samples and samples with known genotype were included as negative and positive controls, respectively. We achieved 100% concordance in the analysis of duplicate samples (6% of total).

Statistical analysis

The HWE program of the LINKUTIL package (downloaded from http://linkage.rockefeller.edu/ott/linkutil.htm) was applied to check for deviations from HWE with the significance level set at 0.01. Three analyses were

performed for each of the selected SNPs to assess their involvement in complex obesity: (1) genotype distribution differences between cases and controls were evaluated by Chi-square analysis; (2) ORs were calculated by univariate logistic regression under an additive, dominant, and recessive model; (3) associations between SNP genotype and five obesity parameters (i.e., waist, WHR, TFA, VFA, and SFA) in obese subjects were tested as follows : the additive model was evaluated with a Kruskal–Wallis test, both on unadjusted values and on studentized residuals after correction for age and BMI. Differences between mean values of the parameters for a dominant and recessive model were assessed by Wilcoxon Rank-Sum tests, both with and without adjustment for age and BMI. For the dominant model, genotypes were recoded to compare wildtype subjects versus subjects with one or two copies of the variant allele. Recoding the genotypes for the recessive model then, allowed comparison of individuals with two copies of the mutant allele versus combined wildtype and heterozygous subjects. Pairwise Pearson correlations were calculated between BMI and TFA, VFA, and SFA. Linear regression was used to adjust parameters for age and BMI. Multiple testing correction was done by controlling the false discovery rate (FDR) according to Benjamini and Hochberg [54]. All statistical analyses were performed using SPSS version 15.0 (SPSS, Chicago, IL, USA).

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