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ABSTRACT

Background: **The Ectonucleotide Pyrophosphatase Phosphodiesterase1 (ENPP1) polymorphisms have been associated with metabolic traits. There is no data on the effect of ENPP1 in South African children or adults.**
***Objective*: To investigate the role of K121Q (rs1044498), rs997509 and rs9402349 in obesity and other components of the metabolic syndrome.**
***Design*: A case-control study.**
***Subjects*: Sixty four obese and 64 lean mixed ancestry learners.**
***Setting*: Western Cape, South Africa.**
Main outcome measure: **The ENPP1 rs997509T allele is independently associated with obesity in children of mixed ancestry from South Africa.**
***Results*: The T allele frequency of the rs997509 differed significantly between obese and controls, p=0.0100 and increased the risk of being obese, p = 0.0238. Furthermore, the estimated effect of the T allele was an increase of 8.6 cm in waist circumference, 10.2 kg in weight and a corresponding 4.9 kg/m2 in BMI. Individuals carrying both the 121Q and the T allele of rs997509 were more associated with obesity (odds ratio = 3.85, 95% CI: 1.13 to 13.09) whilst those carrying the C allele of rs997509 in the presence of 121Q were likely to be lean with odds ratio of obesity 0.41 (95% CI: 0.19 to 0.87).**
***Conclusion*: Our findings suggest that ENPP1 polymorphisms may contribute to different metabolic characteristics, all of which are associated with insulin resistance in mixed ancestry children of South Africa. However, a larger study is required to confirm findings of this study.**

INTRODUCTION

The attention has previously been focused on under-nutrition in South African children, but recent survey data indicate that the country is faced with a rapid increase in childhood obesity of up to 20% among children and adolescents between the ages of 1-19 years old (1-3). A national survey conducted in 10,195 South African 6 - 13 year old primary school learners reported an overweight prevalence of 10.8 and 13.0% in boys and girls respectively (4). Overweight children tend to become overweight adults, and are thus at an increased risk of developing insulin resistance (5, 6), Insulin resistance and obesity are considered to be the driving force of metabolic syndrome (7,8) . Recently, in a study of learners aged 10 - 16 years, we showed that the metabolic syndrome was more prevalent in obese subjects using either the National Cholesterol Education Programme Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults Adult Treatment Panel III (NCEP ATP III) or International Diabetes Federation (IDF) (9).

Multiple studies assessing heritability within families have shown compelling evidence for the significant genetic influence on obesity. Genetic factors are currently estimated to account for approximately 40-70% of the variance in human adiposity (10). Ectonucleotide Pyrophosphatase Phosphodiesterase 1 (ENPP1) is a promising candidate gene for early onset obesity and related sequelae. ENPP1, a class

II transmembrane glycoprotein has been shown to inhibit insulin receptor function by decreasing insulin-induced tyrosine phosphorylation in peripheral tissues, including liver, muscle and fat (11, 12). While the physiological function of ENPP1 is not well characterised, studies in humans have shown a correlation between over expression of ENPP1 and insulin resistance, obesity, and type 2 diabetes, particularly the substitution of a lysine with a glutamine at codon 121 (K121Q) (13-15). Recently, another Single Nucleotide Polymorphism (SNP) within K121Q haploblock, rs997509 has been associated with diabetes and metabolic abnormalities in obese children (16,17). The rs997509 is located in the 3' end of intron 1 and consists in a substitution of a cystosine (C) with a thymine (T).

There is currently no data showing the contribution of genetic factors to childhood obesity in South African children and adolescents. The aims of our study were to genotype the K121Q (rs1044498), rs997509 and rs9402349 variants among mixed ancestry obese and normal weight children from South Africa, and to establish their role in obesity and other components of the metabolic syndrome. The mixed ancestry population is the majority group in the Western Cape, South Africa (18).

MATERIALS AND METHODS

Ethics Statement: Ethical Approval was obtained from the Research Ethics Committee of the University of Stellenbosch (Project number: N07/07/ 160) and from the Faculty of Health and Wellness Science Ethics Committee, Cape Peninsula University of Technology (Project Reference number: CPUT/ HAS-REC 0016). Written consent was obtained from the parent or guardian of each learner, and oral assent from children was obtained on the sampling day.

Study population: In an epidemiological study that aimed to determine the prevalence of metabolic syndrome and obesity, learners were recruited through a proportionally stratified multistage random sampling technique from government funded primary and secondary schools (9). Of the 1564 learners that responded (65%), the 861 (55.1%) were of mixed ancestry. Obesity was assessed using gender-age specific cut-off points international references provided by the International Task Force as developed by Cole and his co-workers (19). The 64 (7.4%) that were found to be obese and 64 normal weight unrelated mixed ancestry learners, all consented for further genetic investigations. Anthropometric measurements (body weight and height; waist and hip circumference; waist-hip ratio and skinfold thickness measurements etc), were performed on all learners. Body Mass Index (BMI) was calculated as weight per square meter (kg/ m^2). Three readings were taken for blood pressure, waist and hip circumferences. Skinfold thickness was measured at three different body sites, namely subscapular, supra-ileac and upper arm.

Finger prick blood was used for the estimation of fasting plasma glucose and lipid levels using the Accutrend GCT glucometer and CardioCheck TM, P.A analyser (Polymer Technology Systems, Inc, USA), respectively. Blood pressure measurements were performed using a semi-automatic digital blood pressure monitor (Rossmax PA, USA) according the WHO guidelines (20).

Genotyping of the ENPP: Genomic DNA was extracted from either whole blood collected in vacutainer Ethylenediamine Tetra-acetic Acid (EOTA) tubes, or from capillary blood collected onto Whatman FTA® Cards (Merck Laboratories, United Kingdom), All learners were genotyped for the K121Q (in exon 4), rs997509 (at the 3' end of intron 1) and rs9402349 (in intron 9) SNP's by PCR and subsequent sequencing. The following primers were used for amplifying the respective fragments,: K121Q, F: 5'- ACTTTGGACATGTTGAATTTGAGAC-3' and R:5'-ACACACAGAACTGTAGTTGATGCAG-3'; rs997509, F:-5'CCTTCAGTGTATAACAGTCT TTGC-3' and R: 5'-CCCATTCTCCACTCTTCTGG-3'; rs9402349, F: 5'-TTCCTCTGGACA CAGGCTTT- 3' and R: 5'- GAGGTGGAGATTGCAGTGAA-3'. All primers were designed using Primer3plus (http: www.primer3plus.com). Sequencing was done using the BigDye Terminator. Sequence Ready Reaction Kit version 3.1 (Applied Biosystems) and the products electrophoretically separated on a 3130xl Genetic Analyzer (Applied Biosystems). The sequencing data were analysed using the Sequencing Analysis version 5.2 (Applied Biosystems) and Bioedit (21) software programmes.

Statistical Analysis: Data were analysed using the freely available programming language R (www.r-project.org) and specifically packages DGC-genetics (LD, Hardy-Weinberg, genotype and allelic association) and haplo.stats (inferred haplotype association), Linear models were used to examine differences in traits that were measured once only, with added random effects (linear mixed-effects models) for the traits where measurements were replicated (Midupperarm, waist circumference, blood pressure and others). All models were adjusted for age and gender, while those for midupperarm were also adjusted for site of measurement. Effect estimates are derived from the same models. Because only one T/T homozygote was observed for rs997509, it was decided to combine it with the C/T heterozygote and analyse it as a dichotomous genotype for estimating the effect of the T allele. No rare-allele-homozygotes, G/G, were observed for rs9402349.

Genotype-association (2 degrees of freedom) was tested by coding genotype as a categorical factor and additive allelic association (1 degree of freedom) with a numerical variable, counting the number of smaller-frequency alleles. For rs9402349, because it is dichotomous, the analysis is the same for genotype as for allelic association. We inferred haplotypes from the genotypes of the three variants and compared frequencies of obese and control subjects.

RESULTS

The clinical characteristics of 64 obese cases and 64 controls are summarised in Table 1. Significant differences between obese and control subjects were noted for fasting blood glucose, diastolic blood pressure and HDL cholesterol levels after adjusting for gender. HDL cholesterol also differed significantly between the genders, after adjusting for body size. Females had higher HDL-cholesterol levels, waist/hip ratios, and sum of skinfolds after adjusting for gender and repeated measures. The genotype distributions of all variants studied were in Hardy-Weinberg equilibrium in both obese and control subjects. K121Q and rs997509 showed tight linkage disequilibrium in the cases (D′ = 0.999, P < 0.01), whereas K121Q and rs9402349 showed tight linkage disequilibrium in the controls (D′ =1.0, P = 0.02). The observed differences in allelic distribution of K121Q and rs9402349 did not differ significantly, whilst that of rs997509 did differ significantly between obese and controls (p=0.0100). The effect of the of rs997509 T allele was studied after adjustments for age and gender and was found to significantly increase the risk of being obese, p = 0.0238. The significance of this association was independent of the effects of the other two SNPs (Table 2).

Table 1

Characteristics of cohort, stratified by age and status. Values are mean ± standard deviation. P-values are from the same model - adjusted for each other, (P-value for equality of status groups is adjusted for gender and vice versa

	Obese		Norml weight			
	Female	Male	Female	Male	p-value	
No	48	16	48	16	status	Gender
Age (years)	13.2±2.7	13.2±2.6	12.9±2.7	13.2±2.7	0.6442	0.8192
Weight (kg)	75.5±15.7	78±20.9	40.7±10.6	39.8±10.7	<0.0001	0.7989
Height (cm)	154.7±10.4	157.3±14	147.1±11.5	150.5±14.3	<0.0001	0.2134
BMI (kg/m^2)	31.2 ±4	30.9±4.3	18.4±2.8	17.2±1.8	<0.0001	0.2788
Midupperarm (cm)*	29.9±3.7	31.3±3.7	21.3±2.6	20.2±2.6	<0.0001	0.7172
WC (cm)*	88.1±10.2	91.9±10.3	62.5±7.4	60.4±5.7	< 0.0001	0.6354
HP (cm)*	107.4±11.5	107.9±14.3	81.8±10.3	75.7±7	< 0.0001	0.2798
WHR (ratio)*	0.823±0.061	0.855±0.051	0.77±0.047	0.791±0.025	< 0.0001	0.0057
Skinfold (cm)**	2.27±0.99	2.29±0.91	0.89±0.33	0.53±0.14	< 0.0001	0.0478
SBP (mm Hg) *	111.8±12.2	117.6±15.6	109.4±66.9	102.4±14.2	0.2050	0.9076
DBP (mm Hg)*	69.3±10.3	70.7±12.4	66.1±11.3	59.3±12.2	0.0017	0.1484
FBG (mmol/L)	4.25±0.66	4.74±0.76	4±0.71	4.03±0.89	0.0049	0.0871
TC (mmol/L)	3.7±0.71	3.42±0.67	3.77±1.16	3.39±0.85	0.7831	0.0776
TG (mmol/L)	0.796±0.305	0.919±0.428	0.795±0.532	0.767±0.248	0.5979	0.5739
HDL-C (mmol/L)	0.931±0.334	0.718±0.204	1.137±0.382	0.976±0.316	0.0004	0.0074
LDL-C (mmol/L)	2.42±0.58	2.58±0.7	2.48±1.52	2.15±0.7	0.7430	0.7443

* replicated measurements; ** measured at 3 different sites; WC waist circumference; HP, hip circumference; WHR, waist hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBG, fasting blood glucose; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol.

Table 2

Allelic and genotype counts (n), frequencies (f) and p-values for corresponding tests of association with obesity status. Adjusted p-values are all from the same model; each is adjusted for the effect of the other two variants.

	Obese		Normal			Adjusted
	No	f	No	f	P-value	p-value
rs997509	(n = 24)		(n = 33)			
C	37	0.77	62	0.94		
T	11	0.23	4	0.06	0.0100	
C/C	14	0.58	29	0.88		
C/T	9	0.38	4	0.12		
T/T*	1	0.04	0	0	0.0337	0.0238
rs1044498	(n = 61)		(n = 57)			
C	64	0.52	68	0.6		
A	58	0.48	46	0.4	0.2445	
C/C	20	0.33	20	0.35		
A/C	24	0.39	28	0.49		
A/A	17	0.28	9	0.16	0.3033	0.1790
rs9402349	(n = 59)		(n = 42)			
T	109	0.92	76	0,9		
G	9	0.08	8	0.1	0.7276	
T/T	50	0.85	34	0.81		
T/G	9	0.15	8	0.19	0.7276	0.7041

* One T/T homozygote observed for rs997509, was combined with the T/C heterozygote and analysed as a dichotomous genotype for estimating the effect of the T allele.

The SNP rs997509 was significantly associated with weight, BMI, skinfold thickness, midupperarm, waist and hip circumferences (Table 3a). The estimated effect of the T allele was an increase of 0.64 cm in skinfold thickness, 3.0 cm in midupperarm circumference, 8.6 cm in waist circumference, 8.9 cm in hip circumference, 10.2 kg in weight and a corresponding 4.9 kg/m2 in BMI respectively, (Table 3b). The most frequent rs997509-K121Q-rs9402349 haplotype was CAT, observed in more than 40% of normal and obese subjects. Haplotype TCT was significantly more frequent in obese (25%) than control subjects (5%) with odds ratio = 4.24 (95% CI: 1.24 to 14.53) compared to CAT, whilst CCT was significantly more prevalent in controls (45%) than obese subjects (24%) with odds ratio of obesity 0.42 (95% CI: 0.19 to 0.98) compared to CAT (Table 4). As haplotypes containing rs9402349 G were very rare ($f < 0.05$), we present an analysis of the rs997509-K121Q haplotype in Table 5. Individuals carrying both the 121Q and the T allele of rs997509 were more associated with obesity (odds ratio = 3.085, 95% CI: 1.13 to 13.09) whilst those carrying the C allele of rs997509 in the presence of 121Q were likely to be lean with odds ratio of obesity 0.41 (95% CI: 0.19 to 0.87).

Table 3a

*P-values for genotype association tests with clinical and metabolic syndrome characteristics, adjusted for age, gender and replicated measurements (marked *). Skinfold was also adjusted for the fact that the folds were measured at different sites. Sizes of significant effects and their standard errors are in Table 3 b.*

	Genetic association		
Characteristic	rs997509	K121Q	rs9402349
Weight (kg)	0.0366	0.5267	0.6905
Height (cm)	0.9896	0.8479	0.8199
BMI (kg/m^2)	0.0090	0.5810	0.6666
Midupperarm (cm)	0.0220	0.5356	0.9225
WC (cm)	0.0347	0.3683	0.9542
HP (cm)	0.0379	0.2690	0.5212
WHR ratio	0.3861	0.8439	0.1076
Skinfold (cm)	0.0068	0.5095	0.9149
SBP (mm Hg)	0.7793	0.4153	0.9588
DBP (mm Hg)	0.3542	0.6308	0.9556
FBG (mmol/L)	0.2660	0.7462	0.3339
TC (mmol/L)	0.2775	0.3379	0.4367
TG (mmol/L)	0.9231	0.9890	0.4977
HDL-C (mmol/L)	0.8805	0.0581	0.4309
LDL-C (mmol/L)	0.7361	0.4638	0.5414

Table 3b

Estimated sizes of effect of T. allele of rs997509 and their standard errors for significant associations

Trait	Effect	Standard error
Weight (kg)	10.2	4.9
BMI (kg/m^2)	4.6	1.7
Midupperarm (cm)	3.0	1.3
WC (cm)	8.6	4.0
HP (cm)	*8.9*	*4.2*
Skinfold (cm)	0.64	0.23

Table 4

Inferred haplotype frequencies in obese and control subjects, in decreasing order of pooled frequency.

	Haplotype		Frequency	
rs997509	rs1044498	rs9402349	Control	Obese
C	A	T	0.40	0.44
C	C	T	0.45	0.24
T	C	T	0.05	0.25
C	C	G	0.08	0.05
C	A	G	<0.01	0.03
T	C	G	0.01	<0.01
T	A	G	<0.01	<0.01
T	A	T	-	<0.01

Table 5
Association of inferred rs997509 and K121Q haplotype with obesity. Odds ratios are relative to CA

Haplotype		Frequency		Odds ratio		
rs997509	K121Q	Control	Obese	OR	95% CI	
C	C	0.54	0.29	0.41	0.19	0.87
C	A	0.40	0.47	1.00		
T	C	0.06	0.24	3.85	1.13	13.09
T	A	–	–	–	–	

DISCUSSION

In this study we provide evidence that the T allele or ENPP1 rs997509 is strongly associated with obesity in mixed ancestry children from the Western Cape, South Africa. Moreover, we show that the estimated effect of the *T. allele* was an increase of 10.2 kg in weight and a corresponding 4.9 kg/m^2 in BMI. Previously, the rs997509 *T. allele* has been associated with diabetes and metabolic abnormalities in obese children (16,17). In contrast, Bochenski *et al.,* did not find an association between the rs997509 *T. allele* and obesity in Polish Caucasians. The haplotype carrying the minor *T. allele* of rs997509 and 121 Q was particularly associated with type 2 diabetes among obese subjects possibly due to the presence of rs997509 *T. allele* (16). Recently, Santora *et aI.,* reported a strong association between rs997509 variant and insulin resistance, metabolic syndrome and IGT in obese children and adolescents from Naples, Italy (17). The glucose levels were significantly higher in obese learners, we did not find any association between the rs997509 *T. allele* and glucose levels, our findings suggest that the *T. allele* of rs997509 variant may predispose individuals to different metabolic syndrome traits according to population groups. The ENPP1 K121Q polymorphism that had been strongly associated with metabolic traits had also shown different outcomes regarding its effect on insulin resistance, type 2 diabetes and obesity in different populations, for example, this variant was associated with type 2 diabetes in Caucasians living in the United States (22, 23) and Finland (24), but not in Swedish Caucasians (25).

Another observation in this study was that the rs997509 T allele also showed strong associations with other indices of obesity. The waist circumference is commonly used as an indicator of central obesity, and its metabolic consequences, insulin resistance and type 2 diabetes (26). Recognition of distinct gender and ethnic differences in body fat distribution has led to the use of different waist circumference cut-off points in the classification of central obesity (26, 27). The mixed ancestry population of South Africa is a combination of European settlers and the indigenous Africans. Though these associations may be unique to this population group, they buttress the effect of rs997509 on obesity and indirectly on metabolic syndrome traits.

Similar to observations reported in African-Americans, the ENPP1 121Q allele frequency was high in our cohort (28,29). The 121Q allele frequency has been shown to differ greatly between ethnic groups with the least number of carriers amongst Caucasians and higher numbers in Asian Indians and African-Americans (22, 23, 28, 29). Though the African-Americans have been shown to have the highest 121Q allele frequency, no associations with BMI or obesity were demonstrated in previous studies (28, 29). Likewise, in this study we did not find any association between K121Q and obesity. Individuals carrying the 121Q and the T allele of rs997509 were more associated with obesity whilst those carrying the C allele of rs997509 in the presence of 121Q were likely to be lean. These findings seem to indicate that 121Q does not contribute to obesity and that the effect of the T allele of rs997509 is independent. On the other hand both co-existence of the T allele of rs997509 and K121 were rare in our study group, with inferred frequencies below 1%. It is likely that the TA haplotype does not exist in this population at all.

The major limitation of this study is the small sample size, also the SNPs could not be genotyped in all individuals due to the limited amount of DNA obtained from the FTA card as venous blood was only collected in learners aged ≥16 years. This could result in us not having enough power to detect significant differences. Furthermore, this small sample could not allow for any sub-grouping such as gender-specific analysis, gender and ethnic differences in the ENPP1 region have been reported(22,23,28,29,30). Therefore, these preliminary findings should be interpreted with caution, however they warrant further investigation on a larger samples size. In conclusion, our results concur with previous reports that ENPP1 variants contribute to obesity. Our findings suggest that ENPP1 polymorphisms may contribute to different metabolic characteristics, all of which are associated with insulin resistance in mixed ancestry children of South Africa.

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