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eNOS polymorphism associated with metabolic syndrome in children and adolescents

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Abstract We investigated whether genetic polymorphisms in the endothelial nitric oxide (eNOS) gene ($T^{786}C$ in the promoter region, Glu298Asp in exon 7, and 4b/4a in intron 4) or eNOS haplotypes are associated with metabolic syndrome (MetS) in obese children and adolescents. We studied 242 subjects: 108 healthy (controls), 64 normotensive obese, and 70 obese children and adolescents with MetS. Genotypes were determined by Taqman® allele discrimination assay and real-time polymerase chain reaction (PCR), and PCR followed by fragment separation by electrophoresis. We compared the distribution of eNOS genotypes, alleles, and haplotypes in the three groups of subjects. The CC genotype for the $T^{786}C$ polymorphism was more common in the MetS group than in the control group (OR = 3.27; CI 1.81–9.07; $P < 0.05$). However, we found no significant differences in the distribution of eNOS haplotypes ($P > 0.00625$; P for significance after correction for multiple comparisons). Our findings suggest that while eNOS haplotypes are not relevant, the CC genotype for the $T^{786}C$ polymorphism is associated with MetS in obese children and adolescents. Further studies examining interactions of eNOS haplotypes with environmental factors and other genetic markers are warranted.

Keywords Adolescents · Children · Endothelial nitric oxide synthase · Obesity · Metabolic syndrome · Polymorphism · Haplotype

Introduction

Obesity in childhood has reached epidemic proportions worldwide, and it is associated with increased prevalence of metabolic syndrome (MetS) [1]. MetS is characterized by a group of cardiovascular risk factors including visceral obesity, hypertension, dyslipidemia, glucose intolerance, and insulin resistance [2, 3]. A common feature of MetS components is the presence of endothelial dysfunction characterized by reduction of the nitric oxide (NO) bioavailability [4–6].

NO is produced in endothelial cells and platelets by endothelial nitric oxide synthase (eNOS), and is very important to maintain vascular homeostasis, to prevent platelet and leukocyte adhesion, and to inhibit vascular smooth muscle cell migration and proliferation [7]. Indeed, experimental studies showed that eNOS gene deletion promotes hypertension and is associated with other cardiovascular risk factors frequently found in humans with MetS such as insulin resistance, dyslipidemia, hyperuricemia, and increased fibrinogen and leptin levels [8, 9]. Importantly, recent clinical studies have shown that functional polymorphisms or haplotypes in the gene encoding eNOS are associated with increased susceptibility to hypertension [10–13], insulin resistance, type 2 diabetes

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mellitus [14], and MetS [15, 16]. However, no previous study has examined the possible interaction of eNOS gene polymorphisms or haplotypes with MetS in children and adolescents. This interaction is relevant and although MetS is directly linked to obesity in childhood, this population is exposed to environmental factors for shorter periods of time as compared with adult populations, and therefore the effects of eNOS genotypes or haplotypes on the susceptibility to MetS should be more easily detected in children and adolescents than in adults.

In this study we aimed at investigating whether eNOS gene polymorphisms or haplotypes (combinations of genetic markers) are associated with susceptibility to MetS in children and adolescents. We studied three functional eNOS polymorphisms that are known to affect NO formation [17, 18]: a single-nucleotide polymorphism (SNP) in the promoter region ($T^{786}C$, rs 2070744), an SNP in exon 7 ($G^{894}T$, rs 1799983), and the variable number of tandem repeats (VNTRs) in intron 4. Our hypothesis is that the eNOS polymorphisms and their haplotypes are associated with MetS in children and adolescents.

Methods

Subjects

Approval for use of human subjects in this study was obtained from the Institutional Review Board at the Federal University of Juiz de Fora, Brazil. Parents and children were informed as to the nature and purpose of the study. Parents gave their written consent and children gave their verbal consent. We studied 64 normotensive obese, and 70 obese subjects with MetS recruited from the Endocrinology Ambulatory of the Adolescent and Child Institute at Juiz de Fora and from the Childhood Endocrinology Ambulatory of the IMEPEN Foundation at Juiz de Fora. The control group consisted of 108 healthy children and adolescents recruited from the local community. All children underwent thorough physical examination. Height was measured to the nearest 0.1 cm using a wall-mounted stadiometer.

The body weight was measured with a digital scale to the nearest 0.1 kg. Body mass index was calculated as the weight in kilograms divided by height in meters squared. Obesity was defined as body mass index greater than the 95th percentile, matched according to age and sex [19]. The modified definition of MetS from the NCEP ATP III (MS-ATP) was used, with the presence of at least three of the following criteria: abdominal obesity, arterial hypertension, increased triglycerides, decreased HDL, and glucose intolerance [20, 21]. The waist circumference measurement was made at the midpoint between the bottom of the rib cage and above the top of the iliac crest. Systolic (SBP) and diastolic blood pressures (DBP) were measured at least three times and the presence of hypertension was defined as SBP and/or DBP exceeding the 95th percentile [22].

At the time of clinic attendance, venous blood samples were collected and genomic DNA was extracted from the cellular component of 1 ml of whole blood by a salting-out method and stored at −20 °C until analyzed [13].

Laboratorial analyses

Glucose concentrations and lipid parameters (total cholesterol, triglycerides, and high-density lipoprotein cholesterol) were determined in plasma and serum, respectively, with routine enzymatic methods using commercial kits (Labtest Diagnostic, SA, Lagoa Santa, Brazil). Low-density lipoprotein concentration was calculated according to the Friedewald formula [23]. Insulin concentrations were measured in EDTA–plasma using a kit (Genese Diagnostics Products, Sao Paulo, Brazil). The insulin resistance was determined by applying the evaluation model homeostatic sensitivity to insulin (HOMA-IR), as described by Matthews et al. [24].

Genotype determination

Three clinically relevant polymorphisms of eNOS gene were studied: $T^{786}C$ (rs 2070744), polymorphism in the 5′-flanking region of eNOS gene, VNTRs (27-bp repeat) polymorphism in intron 4, and the $G^{894}T$ (rs 1799983) polymorphism in exon7. Genotypes for the $T^{786}C$ and for the Glu298Asp polymorphisms were determined by Taqman Allele Discrimination assay and real-time PCR on Chromo 4 Detector (Bio-Rad Laboratories, Hercules, CA, USA). Genotypes for the VNTR polymorphism in intron 4, however, were determined by PCR and fragment separation by electrophoresis in 8 % polyacrylamide gels as previously described [13].

Statistical analysis

The clinical characteristics of MetS group and normotensive obese were compared with those of control children and adolescent by one-way ANOVA followed by Tukey post-hoc. The categorical variables were compared between groups by χ^2 tests. The distribution of genotypes for each polymorphism was assessed for deviation from the Hardy–Weinberg equilibrium, and differences in genotype frequency and in allele frequency between groups were assessed using χ^2 tests. A value of $P < 0.05$ was considered statistically significant. Haplotypes were inferred using the Bayesian statistical based program PHASE version 2.1 (http://www.stat.washington.edu/stephens/software.html)

to estimate the haplotype frequencies. The possible haplotypes including genetic variants of three polymorphisms in the eNOS gene studied ($T^{786}C$, intron 4, and Glu298Asp) were H1 (T b Glu), H2 (T b Asp), H3 (C b Glu), H4 (C b Asp), H5 (T a Glu), H6 (T a Asp), H7 (C a Glu), and H8 (C a Asp). Differences in haplotype frequency were further tested using a contingency table, and a value of $P < 0.00625$ (0.05/number of haplotypes − 8) was considered significant to correct for the number of comparisons made.

Results

The clinical and laboratorial characteristics of the studied groups are presented in Table 1. As expected, the obese and MetS group subjects presented higher body mass index and waist circumference than the control group ($P < 0.05$; Table 1). Compared to the control group, the obese group presented higher total cholesterol, low-density lipoprotein, and HOMA-IR ($P < 0.05$; Table 1). The MetS group presented higher age, systolic blood pressure, diastolic blood pressure, total cholesterol, low-density lipoprotein, triglycerides, glucose, insulin, and HOMA-IR, when compared to the control group (all $P < 0.05$). High-density lipoprotein concentrations were higher in the control group than in the MetS group ($P < 0.05$).

Table 2 shows the distribution of eNOS genotypes and alleles in the three study groups. The genotypes distribution for each polymorphism did not present Hardy–Weinberg deviation (all $P > 0.05$). Since there are interethnic differences in the distribution of eNOS polymorphisms [25, 26], we carried out two different analyses. The first analysis included white and black children and adolescents, whereas the second analysis took into consideration only white children and adolescents, which corresponded to 50 % of the subjects. However, the results were similar to those found in the first analysis (data not shown).

We found no significant associations between eNOS polymorphisms and obesity, except for the ^{289}Asp/Asp genotype, which was more commonly found in controls than in obese children ($P < 0.05$; Table 2). However, the odds ratio was not significant (OR = 0.08; CI 0.01–1.47; Table 2). Conversely, the $T^{786}C$ polymorphism was found in association with MetS. The ^{786}CC genotype was more common in the MetS group than in the control group (15.7 % vs 6.5 %, respectively; OR = 3.27; CI 1.81–9.07; $P < 0.05$; Table 2).

The analysis of eNOS haplotypes showed no significant differences between groups (all $P > 0.00625$, which is the P value corrected for multiple comparisons; Table 3).

We separated girls ($N = 139$) and boys ($N = 103$) and carried out additional analysis shown in Supplemental Tables 1 and 2. While the CC genotype for the $T^{786}C$ polymorphism was more common in controls than in obese girls ($P = 0.017$; Supplemental Table 1), the 4a4a genotype for the polymorphism in intron 4 was more common in girls with MetS than in controls ($P = 0.031$; Supplemental Table 1), without significant differences when eNOS haplotypes were taken into consideration. However, we found that the TC and the CC genotypes, or the C allele for the $T^{786}C$ polymorphism were more common in obese or in MetS boys than in controls (all $P < 0.01$; Supplemental Table 2). In addition, we found that the H5 (C-4b-Glu) haplotype was more common in MetS boys than in controls (15.5 vs 2.7 %; $P = 0.005$; Supplemental Table 2), whereas the H2 haplotype (T-4b-Asp) haplotype was more common in controls than in MetS boys (12.2 vs 0 %; $P = 0.001$; Supplemental Table 2).

Table 1 Demographic characteristics of study participants

Parameters	Control	Obese	MetS
N	108	64	70
Age (years)	12.7 ± 2.5	10.5 ± 2.0*	11.6 ± 2.3*
BMI (kg/m^2)	18.8 ± 2.8	24.7 ± 4.6*	29.5 ± 4.1*
WC (cm)	68.9 ± 9.5	82.3 ± 14.0*	97.7 ± 10.5*
SBP (mmHg)	106.5 ± 10.2	107.7 ± 9.3	123.8 ± 13.2*
DBP (mmHg)	65.8 ± 9.2	68.2 ± 8.8	77.5 ± 8.4*
Glucose (mg/dL)	81.5 ± 10.8	84.4 ± 9.9	87.5 ± 9.6*
Total cholesterol (mg/dL)	131.4 ± 28.3	139.5 ± 30.9	157.3 ± 40.1*
LDL (mg/dL)	71.0 ± 28.5	79.5 ± 33.5	100.2 ± 37.1*
HDL (mg/dL)	43.9 ± 9.0	41.8 ± 9.5	33.5 ± 8.8*
Triglycerides (mg/dL)	72.3 ± 26.1	70.3 ± 27.4	115.0 ± 48.0*
Insulin (mμI/mL)	19.3 ± 8,5	21.8 ± 10.8	32.5 ± 12.2*
Homa-IR	3.9 ± 1.9	4.6 ± 2.4	7.1 ± 3.1*

Values are the mean ± SD

BMI body mass index, *WC* waist circumference, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *HOMA-IR* homeostatic model assessment-insulin resistance

* $P < 0.05$ versus control

Discussion

This study was the first to assess the possible association between eNOS polymorphisms and haplotypes with MetS in children and adolescents. Although the main finding of the present study was that the ^{786}CC genotype is associated with MetS in children, the analysis of eNOS haplotypes showed no significant associations with obesity of MetS. Our findings suggest that the biological variations associated with the $T^{786}C$ polymorphism predispose to MetS in children and adolescents.

Table 2 Genotype and allele frequencies of the endothelial nitric oxide synthase polymorphism in controls, obese, and MetS children and adolescents

Genotype	Control (108)	Obese (64)	OR (95 % CI)	*P* value	MetS (70)	OR (95 % CI)	*P* value[a]
$T^{786}C$							
TT	0.537 (58)	0.391 (25)	1.00 (reference)	–	0.443 (31)	1.00 (reference)	–
TC	0.398 (43)	0.515 (33)	1.80 (1.00–3.22)	ns	0.400 (28)	1.22 (0.68–2.22)	ns
CC	0.065 (7)	0.094 (6)	2.08 (0.68–6.31)	ns	0.157 (11)	3.27 (1.81–9.07)	0.032
T allele	0.736 (159)	0.648 (83)	1.00 (reference)	–	0.643 (90)	1.00 (reference)	–
C allele	0.264 (57)	0.352 (45)	1.53 (0.83–2.81)	ns	0.357 (50)	1.60 (0.87–2.93)	ns
Intron 4							
4b,4b	0.593 (64)	0.594 (38)	1.00 (reference)	–	0.557 (39)	1.00 (reference)	–
4b,4a	0.380 (41)	0.375 (24)	1.00 (0.56–1.78)	ns	0.343 (24)	0.94 (0.52–1.70)	ns
4a,4a	0.018 (2)	0.031 (2)	1.50 (0.24–9.31)	ns	0.086 (6)	4.74 (0.98–22.92)	ns
4a,4c	0.009 (1)	0.000 (0)	0.33 (0.01–8.35)	ns	0.014 (1)	1.05 (0.06–17.27)	ns
b allele	0.782 (169)	0.781 (100)	1.00 (reference)	–	0.729 (102)	1.00 (reference)	–
a allele	0.213 (46)	0.219 (28)	1.05 (0.53–2.06)	ns	0.264 (37)	1.32 (0.69–2.55)	ns
c allele	0.005 (1)	0.000 (0)	0.33 (0.13–8.32)	ns	0.007 (1)	1.07 (0.66–17.41)	ns
Glu289Asp							
Glu/Glu	0.620 (67)	0.594 (38)	1.00 (reference)	–	0.671 (47)	1.00 (reference)	–
Glu/Asp	0.324 (35)	0.406 (26)	1.35 (0.75–2.41)	ns	0.315 (22)	0.92 (0.51–1.69)	ns
Asp/Asp	0.056 (6)	0.000 (0)	0.08 (0.01–1.47)	0.015	0.014 (1)	0.15 (0.02–1.31)	ns
Glu allele	0.782 (169)	0.797 (102)	1.00 (reference)	–	0.829 (116)	1.00 (reference)	–
Asp allele	0.218 (47)	0.203 (26)	0.89 (0.45–1.75)	ns	0.171 (24)	0.73 (0.36–1.47)	ns

CI confidence interval, *ns* not significant, *OR* odds ratio

[a] MetS compared with controls

Table 3 Estimated haplotypes frequencies in controls, obese, and in MetS children and adolescents

Haplotype		Control (108)	Obese (64)	OR (95 % CI)	*P* value	MetS (70)	OR (95 % CI)	*P* value[a]
H1	T b Glu	0.500 (108)	0.516 (66)	1.00 (reference)	–	0.471 (66)	1.00 (reference)	–
H2	T b Asp	0.083 (18)	0.023 (3)	0.24 (0.05–1.19)	ns	0.014 (2)	0.13 (0.02–1.11)	ns
H3	T a Glu	0.148 (32)	0.117 (15)	0.77 (0.32–1.81)	ns	0.157 (22)	1.14 (0.51–2.55)	ns
H4	T a Asp	0.005 (1)	0.000 (0)	0.32 (0.01–8.06)	ns	0.000 (0)	0.35 (0.01–8.92)	ns
H5	C b Glu	0.065 (14)	0.078 (10)	1.09 (0.37–3.26)	ns	0.114 (16)	1.67 (0.60–4.67)	ns
H6	C b Asp	0.134 (29)	0.164 (21)	1.18 (0.52–2.71)	ns	0.129 (18)	1.06 (0.45–2.53)	ns
H7	C a Glu	0.065 (14)	0.094 (12)	1.24 (0.43–3.57)	ns	0.079 (11)	1.02 (0.41–3.62)	ns
H8	C a Asp	0.000 (0)	0.008 (1)	2.89 (0.11–72.56)	ns	0.036 (5)	9.57 (0.50–182.70)	ns

CI confidence interval, *ns* not significant, *OR* odds ratio

[a] MetS compared with controls

The $T^{786}C$ polymorphism has functional implications that results in variable endogenous NO formation, and the C allele was shown to reduce eNOS transcriptional activity by 50 % [27], with impaired shear stress mediated vasodilatation [28]. This particular eNOS polymorphism was associated with insulin resistance in non-diabetic patients and with impaired glycemic control in patients with type 2 diabetes mellitus [29]. Consistent with these previous results, we found a significant association between the ^{786}CC genotype and MetS, and our results suggest that impaired endogenous NO formation associated with the C allele may contribute to MetS in children and adolescents. In line with our results in children, the eNOS $T^{786}C$ polymorphism was shown as a risk factor to MetS in two different adult populations [15, 16]. However, it remains to be determined whether children with the ^{786}CC genotype develop early endothelial dysfunction, which is a common feature of MetS [16], and therefore are prone to early cardiovascular complications during adulthood.

There is strong evidence indicating that eNOS polymorphisms and their haplotypes affect the susceptibility to hypertension and insulin resistance, which are important

factors in the diagnosis of MetS. For instance, data from our group suggest that the eNOS haplotypes are associated with hypertension in obese children and adolescents [30]. This previous finding aligns with studies in adults showing that eNOS haplotypes affect the susceptibility to hypertension and modify the concentrations of markers of NO bioavailability in hypertensive subjects with or without diabetes mellitus [31, 32]. Another study in adults showed significant association between eNOS haplotypes and MetS, thus supporting the idea that genetic variations in the eNOS gene are associated with features of MetS, and may predispose to insulin resistance, hypertriglyceridemia, and low HDL-cholesterol concentrations [33]. Giving further support to this suggestion, eNOS haplotypes were associated with MetS in Arabian adults [16], and in a hypertensive Spanish population [15]. However, no previous study has examined whether these findings in adults are valid in children. In contrast with these adult studies, we no found association between eNOS haplotypes and MetS in obese children and adolescents. This could be explained, at least in part, by the fact that the criteria used for the diagnosis of MetS in children and adolescents may not be the best criteria, although they are widely used by many authors [3, 20, 34]. However, in line with these previous findings, our results in boys showed significant association of the H5 (C-4b-Glu) haplotype with MetS, and therefore it is possible that impaired NO formation associated with this particular eNOS haplotype, independently of ethnicity [17, 18, 35], may contribute to MetS in boys.

Some limitations of the present study should be taken into account. The number of subjects enrolled in the present study may have limited our haplotype conclusions. However, we found a significant association between the $T^{786}C$ polymorphism and MetS. Another possible limiting factor is ethnicity. However, the analysis of all subjects or only white subjects resulted in the same conclusions. Finally, we have not studied cardiovascular events in the subjects included in the present study. This would require a long-term study. However, it is possible that any strategies designed to improve eNOS activity may help to prevent cardiovascular complications associated with genetic markers identified in the present study [36, 37]. Alternatively, diet modifications including possible NO forming substances such as nitrite or nitrate [38, 39] could also help to compensate for impaired endogenous NO production.

In conclusion, our results show a significant association between the ^{786}CC genotype and MetS in children and adolescents. Additional studies to examine the possible interaction between eNOS polymorphisms with environmental factors and other genetic markers involved in the development of obesity and their complications are warranted.

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