Myeloperoxidase 463 G>A and superoxide dismutase Ala16Val gene polymorphisms in obese children

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The aim of the study was to determine the role of MnSOD Ala16Val and MPO G-463A gene polymorphisms in the pathogenesis of metabolic syndrome in obese children.

A total of 97 obese children with insulin resistance and, as a control group, 96 healthy children were enrolled in the study.

In the obese group, AA, AV and VV genotype frequencies of the MnSOD gene and GG, GA and AA genotype frequencies of the MPO gene were not significantly different from the frequencies found in the control group (p=0.555 and 0.530, respectively). In the obese group, children who carry both VV (for MnSOD) and GG (for MPO) alleles (n= 26) had higher HOMA-IR levels (6.51±3.91 vs 5.03±2.12) than those of all other genotype combinations (n=71) (p=0.013).

Children who have the maximum risk of developing oxidative stress with the combination of the VV (for MnSOD) and GG (for MPO) genotypes had higher HOMA-IR levels, suggesting these polymorphisms may lead to insulin resistance.

Key words: manganese superoxide dismutase, Myeloperoxidase, oxidative stress, insulin resistance.

Reactive oxygen species (ROS) are products of some aerobic chemical reactions and have essential biological functions in normal physiology. Nevertheless, the balance between oxidants and antioxidants is critical, and increased production of oxidants or defects in the antioxidant system causes oxidative stress in humans[1]. A low-grade inflammation and oxidative stress are involved in the pathophysiologic mechanisms of the development of serious disorders, such as cardiovascular diseases and diabetes mellitus, in the obese population[1,2]. Furthermore, this low-grade inflammation and oxidative stress exist even in early childhood[3,4].

A catalytic enzyme, myeloperoxidase (MPO), is stored within the azurophilic granules of circulating neutrophils, monocytes and some tissue macrophage populations whose catalytic activity results in the generation of various reactive oxidants and diffusible radical species[5]. In spite of the crucial role of MPO-derived ROS in killing invading pathogen microorganisms, they can also cause host tissue injury through oxidative modification of nucleic acids, lipids and proteins, leading to a wide range of chronic inflammatory diseases[6-8]. It has previously been shown that the polymorphism in the promoter region of the *MPO* gene, –463 G>A, affects MPO activity. The MPO G wild-type allele confers about 25 times higher transcriptional activation compared to the –463 A variant in vitro, and the former has been associated with increased MPO mRNA and protein levels in myeloid leukemia cells[9].

Superoxide dismutases are considered to be

antioxidant enzymes, and manganese superoxide dismutase (MnSOD) appears to be a central player in the redox biology of cells and tissues[10]. The role of MnSOD in the mitochondrial matrix is to convert superoxide to hydrogen peroxide molecules[10]. A polymorphism that causes a change from alanine to valine at the 16th amino acid (ala16val) affects MnSOD enzyme activity. Import of the valine protein was found to be partially arrested in the mitochondrial inner membrane, resulting in 30–40% less active MnSOD protein in the mitochondrial matrix. It has been also reported that the *MnSOD*-ala allele was associated with increased production of MnSOD protein per unit mRNA, indicating a possible imbalance in MnSOD protein production from the MnSOD-val mRNA[11].

In obese children, it has been found that oxidative stress is associated with insulin resistance (IR)[3,12]. Elevated mitochondrial reactive oxygen species have been suggested as playing a causative role in some forms of muscle IR[13]. The protective effect of the antioxidant system against IR has also been demonstrated previously[13]. It has been reported that nutritional or behavioral factors, such as a high-fat diet or a sedentary lifestyle, may lead to oxidative stress and IR in obese populations[13,14]. However, we hypothesized that some genetic factors, such as *MPO* –463 G>A and *MnSOD* Ala16Val gene polymorphisms may also have a role in the development of oxidative stress and IR; therefore, these polymorphisms were investigated in obese children with insulin resistance.

Material and Methods

A total of 97 obese adolescents with insulin resistance (58 girls and 39 boys, at a mean age of 12.83±1.94 years old) and 96 normal-weight adolescents as a control group (64 girls and 32 boys, at a mean age of 12.70±2.16 years old) were enrolled in the study. The obese children did not differ significantly from the normal-weight children in age, gender or pubertal stage. The control group was recruited from among healthy children who had been seen in the pediatric clinics for their routine yearly check-ups. Each participant underwent a detailed physical examination (including evaluation for syndromes and endocrine diseases), as well as a laboratory evaluation. Children whose obesity was the result of a syndromal problem (Prader–Willi, Laurence–Moon–Biedl, etc.) were excluded, as were those whose obesity had an endocrinal cause, e.g., Cushing's Syndrome or hypothyroidism. None of the participants were using medications or had a history or evidence of current metabolic, cardiovascular, respiratory or hepatic disease. Patients taking vitamin and/or mineral supplements were excluded.

Standing height (cm) was measured to the nearest 0.1 cm with a Harpenden fixed stadiometer. Body weight (kg) was measured on a SECA balance scale to the nearest 0.1 kg, with each subject dressed in a light T-shirt and shorts. Obesity was defined as a body mass index (BMI) > 97th percentile, the definition of the International Task Force on Obesity in Childhood and population-specific data[15,16]. The degree of being overweight was quantified by Cole's least mean square method, which normalized the BMI-skewed distribution and expressed BMI as a standard deviation score (BMI-SDS)[17]. Pubertal status was determined by criteria set forth by Tanner[18], and prepubertal children were not included in the study.

Fasting plasma glucose, serum triglyceride, total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) concentrations were measured enzymatically using an autoanalyzer (Olympus 2700, Olympus Medical Systems Corp., Tokyo, Japan). The low-density lipoprotein cholesterol (LDL-C) level was calculated using the Friedewald equation. Plasma insulin was measured by the electrochemiluminescence immunoassay method using an automated immunoassay analyzer (E170, Roche, Hitachi, Osaka, Japan). Glucose measurements were carried out by means of the photometric hexokinase method using an Advia 1800 chemistry analyzer (Siemens Healthcare Diagnostics, IL, USA). Homeostasis model assessment of insulin resistance (HOMA-IR) index [fasting insulin (μU/ml) x fasting glucose (mg/dl)/405] was used as a surrogate marker of insulin resistance[19,20]. Insulin resistance criteria were HOMA-IR > 5.22 for boys and HOMA-IR > 3.82 for girls (as calculated for Turkish adolescents)[20].

DNA isolation: Blood specimens were collected in tubes containing EDTA, and DNA samples were extracted from whole blood using a salting-out procedure[21].

MnSOD *Ala16Val genotyping:* For amplification of the *MnSOD* Ala16Val polymorphism, the following primers (Invitrogen) were used: 5'-ACC AGC AGG CAG CTG GC GCC GG-3 '; and 5'- GCG TTG ATG TGA GGT TCC AG -3'.

For detection of *MnSOD* Ala16Val, 50-100 ng genomic DNA was amplified with 1x reaction buffer, 3 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 μM each primer and Taq polymerase (Invitrogen) in a 25 μl reaction volume. The polymerase chain reaction (PCR) conditions were: initial denaturation step at 95°C for 5 min followed by 35 cycles at 95°C for 1 min, 61°C for 1 min, 72°C for 2 min and 72°C for 7 min. PCR products were digested with PdiI restriction enzyme (Thermo Scientific) at 37°C overnight and electrophoresed on 3% agarose gels stained with ethidium bromide. Genotypes were determined as VV (107 bp), AV (18, 89, 107 bp) or AA (18, 89 bp) for the polymorphism[22].

Determination of* MPO *-463 G/A polymorphism: The polymorphic site at position -463 of the *MPO* gene was amplified using forward primer (5'-CGG TATAGG CAC ACA ATG GTG AG-3') and reverse primer (5'-GCA ATG GTT CAA GCG ATT CTT C-3') (Invitrogen) as described in the literature. PCR was performed with Taq polymerase (Invitrogen); the cycling condition was 95˚C for 2 min followed by 35 cycles of 94˚C for 30 s, 62˚C for 30 s and 72˚C for 30 s. PCR product was 350 bp. Forty microliters of PCR products were digested with Aci I restriction enzyme (Thermo Scientific) at 37˚C overnight. Fragments were separated using 2% agarose gel. Three possible genotypes were defined by 3 distinct banding patterns: A/A 289 and 61 bp fragments; A/G 289, 169, 120 and 61 bp fragments; and G/G 169, 120 and 61 bp fragments[23].

Statistical Analysis

All statistical analysis was performed using SPSS 15.0 for Windows. The chi-square test was used to compare the frequency of *MPO* 463 G>A and *MnSOD* Ala16Val gene polymorphisms between groups. Student's t-test was used to compare parameters between the control and study groups. One-way ANOVA was used

Table I. Comparison of Demographic and Laboratory Features of the Groups

	Obese group (n=97)	Control group (n=96)	p
Age (years)	12.83±1.94	12.70±2.16	0.676
Gender (female/male)	58/39	64/32	0.297
BMI SDS	2.19±0.33	-0.06±0.85	<0.001
Systolic blood pressure (mmHg)	132.28±20.27	100.89±12.99	<0.001
Diastolic blood pressure (mmHg)	80.67±11.37	64.5241±9.50	<0.001
Glucose (mg/dl)	88.80±7.86	85.42±9.18	0.052
Insulin (μU/ml)	24.60±12.17	10.15±3.2	<0.001
HOMA-IR	5.42 ±2.69	2.14±0.71	<0.001
TC (mg/dl)	156.98±34.57	156.43±25.32	0.925
Triglycerides (mg/dl)	125.46±67.87	77.52±31.63	<0.001
LDL-C (mg/dl)	111.31±34.95	88.82±23.66	<0.001
HDL-C (mg/dl)	41.23±10.01	54.72±13.11	<0.001
MnSOD polymorphism frequencies			0.555
AA	13.4%	12.8%	
AV	36.1%	43.6%	
VV	50.5%	43.6%	
MPO polymorphism frequencies			0.530
GG	46.4%	50%	
GA	47.4%	40.6%	
AA	6.2%	9.4%	

BMI SDS: Body mass index standard deviation score, HOMA-IR: Homeostatic model assessment of insulin resistance, TC: total cholesterol, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol, MnSOD: manganese superoxide dismutase, MPO: myeloperoxidase

Table II. Comparison of Cardiovascular Risk Factors in Polymorphisms of the *MnSOD* Gene in the Obese Group

	VV	AA	VA	p
BMI	31.61±5.56	32.56±4.52	31.02±4.81	0.616
BMI z-score	2.18±0.43	2.27±0.26	2.18±0.20	0.658
Systolic blood pressure	129.86±21.34	137.69±19.21	133.38±19.25	0.447
Diastolic blood pressure	81.02±11.75	75.38±7.76	82.42±11.75	0.168
HOMA-IR	5.90±3.10	5.12±1.66	4.93±2.39	0.262
TC	160.83±25.88	153.07±32.21	153.82±43.82	0.618
Triglycerides	130.19±78.64	141.07±67.63	114.00±52.25	0.393
LDL-C	115.08±24.99	108.47±31.82	107.86±45.06	0.643
HDL-C	41.24±9.91	42.82±8.23	40.67±10.86	0.818

BMI: Body mass index, HOMA-IR: Homeostatic model assessment of insulin resistance, TC: total cholesterol, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol

to compare cardiovascular risk factors such as HOMA-IR, lipid levels, BMI SDSs, and systolic and diastolic blood pressure levels in different genotype groups for both the *MPO* and *MnSOD* genes.

Student's t-test was also used to compare HOMA-IR, lipid levels, BMI SDSs and systolic and diastolic blood pressure levels for the 26 obese children carrying both VV (for the *MnSOD* gene) and GG (for the *MPO* gene) (VV+GG group) with those of the 74 obese children carrying all other allele combinations for these two gene polymorphisms. Finally, multiple regression analysis was performed to investigate the role of these polymorphisms in HOMA-IR. (BMI SDS, age, gender and genotype combinations were added to the model.)

Results

The obese group had higher BMI z-scores, higher HOMA-IR, TC, triglyceride, LDL-C and systolic and diastolic blood pressure levels and lower HDL-C levels than did the controls (Table I).

In the obese group, AA, AV and VV genotype frequencies in the *MnSOD* gene were 13.4%, 36.1% and 50.5% respectively, while in the control group they were 12.8%, 43.6% and 43.6% respectively. There was no statistically significant difference between the study and control groups in terms of genotype (p=0.555). Also, there were no differences between the *MnSOD* genotype groups in cardiovascular risk factors (Table II).

In the obese group, GG, GA and AA genotype frequencies in the *MPO* gene were 46.4%, 47.4% and 6.2% respectively, while in the control group they were 50%, 40.6% and 9.4% respectively. There was no statistically significant difference between the study and control groups in terms of genotype (p=0.530). There were also no differences between the *MPO* genotype groups in cardiovascular risk factors (Table III).

In the obese group, 26 patients carried both the VV and GG alleles, and these patients had significantly higher HOMA-IR than the remaining patients in the group, who carried all other combinations of alleles (n=71). However, lipid levels, BMI SDSs and systolic and diastolic blood pressure levels were not statistically different between the VV+GG group and the group carrying other combinations. The VV+GG group had higher HOMA-IR levels, but this genotype combination had a minor effect on the model (beta: 0.215, p=0.043). Obesity was the major dependent factor influencing HOMA-IR (beta: 0.532, p<0.001) in multiple regression analyses.

Discussion

Even in childhood, obesity is associated with oxidative stress[3,4]. It has been reported that a hypercaloric diet induces oxidative stress in rats, and that exercise may reduce the adverse effects of a hypercaloric diet[24]. But the development of oxidative stress is complex, and eating or exercise habits alone cannot be implicated; therefore, we investigated the frequencies of *MPO* and *MnSOD* gene polymorphisms in obese children. Despite previous studies

Table III. Comparison of Cardiovascular Risk Factors in Polymorphisms of the *MPO* Gene in the Obese Group

	AA	GG	AG	p
BMI	33.83±4.59	31.17±4.62	31.55±5.04	0.452
BMI z-score	2.33±0.24	2.16±0.42	2.20±0.25	0.528
Systolic blood pressure	144.16±13.57	129.22±19.47	133.53±21.42	0.211
Diastolic blood pressure	86.66±15.05	80.22±11.62	80.24±10.60	0.414
HOMA-IR	6.03±1.11	5.54±3.23	5.23±2.30	0.740
TC	147.33±21.59	160.17±41.59	155.51±29.10	0.648
Triglycerides	87.33±9.52	130.92±74.71	125.82±65.28	0.346
LDL-C	105.73±28.87	115.81±42.54	108.18±28.01	0.573
HDL-C	41.60±8.79	41.50±9.88	40.94±10.47	0.964

BMI: Body mass index, HOMA-IR: Homeostatic model assessment of insulin resistance, TC: total cholesterol, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol

demonstrating a high incidence of oxidative stress in obese populations, the frequencies of *MPO* and *MnSOD* gene polymorphisms in the obese group in our study did not differ from those in the control group. This finding indicates that these polymorphisms have a limited effect on the development of oxidative stress in obese individuals compared to that of other factors.

The relation between oxidative stress and insulin resistance is well defined[25-27]. Diamond-Stanic et al.[28] have demonstrated that low-level oxidant stress significantly impairs insulin-stimulated glucose transport activity at all time points, and is associated with inhibition of insulin-stimulated phosphorylation of downstream signaling elements, including phosphotidylinositol-3-kinase, phosphoinositide-dependent kinases, Akt and glycogen synthase kinase 3β (GSK-3β). They have also demonstrated that in the presence of insulin, H_2O_2 decreases total protein expression of IRS-1 at 6 h, and IRS-2 and phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) is transiently increased by H_2O_2 in the presence and absence of insulin. Finally, they concluded that their results indicate that direct in vitro exposure of isolated mammalian skeletal muscle to low-level oxidant stress impairs distal insulin signaling and insulin-stimulated glucose transport activity, due at least in part to a p38 MAPK-dependent mechanism[28]. Furthermore, Hoehn et al.[29] have demonstrated that antioxidant therapy or overexpression of the *MnSOD* gene may improve insulin resistance. Therefore, we suggested that polymorphisms in the *MnSOD* and *MPO* genes may have a role in the development of IR; however, we could not find any relation between *MPO* 463 G>A and *MnSOD* Ala16Val gene polymorphisms and insulin resistance when the former were analyzed individually. Additionally, no statistically significant differences were found between the control and obese groups in terms of genotype frequencies. However, the study demonstrated that the combined presence of genotypes GG for the *MPO* gene and VV for the *MnSOD* gene (GG+VV) has an effect on the development of insulin resistance.

Family history, hypertension, hypercholesterolemia, diabetes mellitus and smoking have traditionally been suggested as risk factors for coronary heart disease. Oxidative stress plays a role in the pathophysiology of hypertension; it is implicated in endothelial dysfunction, hypertrophy, inflammation, apoptosis, migration, fibrosis, angiogenesis and rarefaction, all important processes involved in vascular remodeling in hypertension[30]. Nitric oxide, which has a vasodilator effect, can be rapidly inactivated by reaction with superoxide, leading to the production of the strong oxidant peroxynitrite ($ONOO^-$). This reaction is important in common conditions leading to endothelial and mitochondrial dysfunction, including hypercholesterolemia, hypertension, diabetes and aging, in which vascular production of superoxide is increased. The SODs are a major part of the cellular defense system against superoxide and peroxynitrite[31]. Van der Zwan et al.[32] have demonstrated that myeloperoxidase is positively and independently associated with blood pressure, and this association is strongest in subjects with (hyperglycemia-induced) oxidative

Table IV. Comparison of the GG+VV Group with Other Allele Combinations in the Obese Group in Relation to Cardiovascular Risk Factors

	GG+VV group (n=26)	Other combinations (n=71)	p
BMI z-score	2.15±0.54	2.20±0.24	0.516
Systolic blood pressure	134.24±19.79	124.89±21.62	0.138
Diastolic blood pressure	79.73±11.95	81.01±11.23	0.647
HOMA-IR	6.51±3.91	5.03±2.12	0.013
TC	164.95±25.52	154.56±36.70	0.230
Triglycerides	140.47±91.44	120.89±58.98	0.249
LDL-C	119.31±24.17	108.96±37.36	0.247
HDL-C	42.18±10.28	40.94±9.90	0.621

BMI: Body mass index, HOMA-IR: Homeostatic model assessment of insulin resistance, TC: total cholesterol, LDL-C: low-density lipoprotein cholesterol, HDL-C: high-density lipoprotein cholesterol

stress. Additionally, Liu et al.[33] have reported that in adults, the *MPO* -463 GA/AA genotype is associated with hypertension. Regarding the relation of MnSOD and hypertension, Miranda-Vilela et al.[34] found an association between *MnSOD* Ala9Val gene polymorphism and hypertension. It has been hypothesized that polymorphisms capable of increasing MPO or reducing MnSOD activities may be implicated in the pathogenesis of hypertension in children with metabolic syndrome, but the present study could not find any relation between these polymorphisms and hypertension, even in the GG+VV group.

Despite some positive findings, there were limitations in this study. First of all, the small size of the groups may have been a handicap in demonstrating the effect of the individual gene polymorphisms under investigation on cardiovascular risk factors. Secondly, the study would have demonstrated more had MPO and MnSOD activity been measured and a correlation with IR shown.

In conclusion, the distribution of the frequencies of *MPO* 463 G>A and *MnSOD* Ala 16 Val gene polymorphisms was similar in obese and control pediatric populations. Additionally, no relation was found between these polymorphisms and cardiovascular risk factors in the obese group when the polymorphisms were analyzed separately; however, the GG+VV allele combination may lead to insulin resistance.

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