British Journal of Medicine & Medical Research
15(7): 1-17, 2016, Article no.BJMMR.25399
ISSN: 2231-0614, NLM ID: 101570965

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The Relationship of eNOS, p22 phox, CETP and ESR1 Gene Polymorphisms Related to Endocrine-Metabolic Parameters and Metabolic Syndrome in Postmenopausal Women - A Sample Population Based Study

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Authors' contributions

This work was carried out in collaboration between all authors. Authors OI and DM analyzed data and drafted the manuscript. Authors RR, SV, SO, OP, MC, CH, AS, LS, MG and CD contributed from their field of expertise. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJMMR/2016/25399
Editor(s):
(1) Vijay K. Yadav, Metabolic Research Laboratory, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi, India.
Reviewers:
(1) Jerzy Bełtowski, Medical University, Lublin, Poland.
(2) Adela Sembaj, National University of Cordoba, Argentina.
(3) Ge Wu, Jilin University, China.
Complete Peer review History: http://sciencedomain.org/review-history/14466

Original Research Article

Received 29th February 2016
Accepted 27th April 2016
Published 5th May 2016

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ABSTRACT

Aims: To investigate the endocrine-metabolic changes in postmenopausal women with MetS and examine relationship with the polymorphisms of eNOS-G894T, p22(phox)-930 A/G, CETP TaqIB, ESR1 (PvuII and XbaI) genes.

Methods: 280 postmenopausal apparently healthy women aged between 60 and 80 years were classified into non-MetS (212) and MetS (68). Clinical, anthropometric and endocrine-metabolic parameters were measured. The single nucleotide polymorphisms were determined and tested for interacting with these parameters.

Results: The weight, waist circumference, blood pressure, WBC, triglycerides, LDL-C, TG/HDL-C ratio, apolipoprotein (apo)B, apoB/apoA-I ratio, fasting glucose, insulin, HOMA, uric acid, were higher and HDL-C was lower in MetS group thus fulfilled the criteria for the MetS. The significant higher levels of E2, T3, GHBP, PTH and lower levels of cortisol, SHBG, FSH, LH, IGFBP1, cortisol/DHEA ratio were also detected.

Genetic association studies showed that presence of A allele p22phoxA/G (OR=1.62; CI=1.08-2.42) and heterozygote AG-XbaI(ESR1) (OR=2.29; CI= 1.19-4.37) indicated a significant risk for MetS. The binary logistic regression (MetS vs Controls) showed an interaction of G894TeNOS polymorphism with MetS (OR>2.5; 95% CI =1.47-4.90) that associated with SBP, TG, apoB, uric acid, ASTGOT (OR>1) and HDL-C (OR<1). CETP TaqIB polymorphism associated with MetS (OR<1) in presence of SBP, GLU, TG with OR>1. ESR1 PvuII (T/C) associated with MetS (OR between 1.59-8.60) in presence of LDL-C, TG/HDL-C ratio, P with OD>1 and HDL-C, androstenedione, SHBG, FAI with OR<1.

In MetS group the carriers of -TT (eNOS-G894T) genotype had higher levels of blood pressure, glucose; -GG (p22phox A/G) had higher levels of BMI, apoB/apoA ratio; -B1B2 (CETP B1/B2) had higher levels of SBP, glucose, cholesterol, HDL-C, CRP, GHBP and lower levels of TSH; -CC (PvuII) and GG (XbaI) ESR1 genotypes showed higher levels of glucose.

Conclusions: These results sustain an interaction between the studied polymorphisms and the endocrine-metabolic changes in MetS pathogenesis.

Keywords: Genetic polymorphisms; postmenopausal women; metabolic syndrome; hormonal and metabolic parameters.

1. INTRODUCTION

Aging is a complex, multifactorial process that dramatically influences human health and society. In this context, there is a perceived need to improve the quality of life for elderly population. The age-dependent endocrine changes, regulated by the central nervous system, are associated to dynamics of neuronal behavior, neuro-degeneration, cognition, biological rhythms, sexual behavior, and metabolism processes and also may have a great impact on life span and normal aging. The age of onset and rate of functional decline vary widely among the aging population, consistent to the regulatory role of genetic factors.

Over the past decades epidemiological research of so-called “complex” diseases has identified a number of risk factors for the common age-related disorders. Genetic mechanisms that possibly trigger and define the rate at which we age have been well documented in lower organisms, but less is known in humans. Nowadays, a novel class of risk factors, genetic polymorphisms gained considerable interest. Associated studies have suggested the involvement of candidate genes in age-related disorders. Many of polymorphisms of these genes are associated with alteration of endothelial function such as endothelial NOS (eNOS) gene [1-5], oxidative stress such as p22phox gene-a subunit of NADPH-oxidase [6-9], lipid metabolism such as Cholesteryl Ester Transfer Protein (CETP) gene [10-13]. Also, variants in the estrogen receptor-α (ESR1) gene have been associated with components of the metabolic syndrome (MetS), including obesity, HDL/LDL cholesterol (HDL/LDL-C) metabolism, blood pressure (BP), and type 2 diabetes [14-16].

MetS is a constellation of characteristics that increases the risk for the development of diabetes (tpye 2 diabetes) and cardiovascular disease in postmenopausal women (PM) [17-24]. Many cross-sectional studies have shown an

increased risk of metabolic syndrome in postmenopausal women which prevalence varies from 13.3 to 51% [25-33]. The pathophysiology of MetS is not completely understood, but is thought to involve a complex interaction between the environment, genetic susceptibility, insulin resistance, and abnormal adipose tissue function [34-37].

The purpose of this study is to focus the endocrine-metabolic changes in postmenopausal women with metabolic syndrome and examine relationship with the polymorphisms of eNOS-G894T, p22(phox) (-930 A/G) , CETP TaqIB , ESR1 (PvuII and XbaI) genes. The SNPs (rs1799983, rs9932581,rs708272, rs9340799, rs2234693) of these genes were selected according to published literature that show to be associated with the susceptibility to components of MetS.

eNOS is a constitutive enzyme present in vascular endothelium that catalyzes nitric oxide biosynthesis, the main regulator of the vessel wall. Mice lacking eNOS are affected by insulin resistance, hyperlipidemia, and hypertension. The variant, G894T (rs1799983) of eNOS gene is commonly studied because of its relevance to eNOS activity [1-5]. Previously, we found that the eNOS-G894T polymorphism is associated with higher values of waist circumference, systolic and diastolic blood pressure (SBP/DBP), resistin, cortisol (F), 17-hydroxy progesterone (17OHP), androstenedione(A4), prolactin (PRL) in subjects with MetS (aged 23-60 years) [38].

p22 phox protein is a major component of NADPH oxidase, a critical subunit that plays an essential role in NADPH oxidase activation in vascular cells. The −930A/G (rs9932581) polymorphism of p22 phox gene is located in the promoter region of *CYBA* at position −930 from the ATG codon and was reported to be associated with hypertension [8,39].

CETP mediates the exchange of lipids between anti-atherogenic high-density lipoprotein (HDL) and atherogenic apolipoprotein (apo) B containing lipoproteins and therefore plays a key role in human lipid metabolism. The gene coding for CETP encompasses 16 exons and is localized on chromosome 16q21. It has been demonstrated that the CETP gene is polymorphic and one of the most widely studied polymorphisms being TaqIB (rs708272). Most studies indicate that the TaqIB B1B1 genotype is associated with higher plasma CETP and lower HDL-C levels than the B2B2 genotype [10-14, 40-41].

The polymorphisms, XbaI (rs9340799) and PvuII (rs2234693) mapped in intron 1, are the two most studied polymorphisms of ESR1gene that associated to dyslipidemia, insulin resistance, hypertension, central obesity and type 2 diabetes[15-16,42].

2. MATERIALS AND METHODS

2.1 Study Design and Subjects

Two-hundred-eighty apparently healthy women aged between 60 and 80 years participated. This study was a part of the GENAGE National Project that was first study related to GENETICS AND ENDOCRINE–METABOLIC-COGNITIVE CHANGES IN HUMAN AGING from Romania (2006-2008). Exclusion criteria for all participants were: - Significant medical illness or organ failure, such as uncontrolled hypertension, diabetes, cardiac disease, cerebrovascular disease, chronic obstructive pulmonary disease, kidney and liver disease; - Significant neurologic disease that might affect cognition, such as Alzheimer's disease, stroke, Parkinson's disease, multiple sclerosis; -Significant current psychiatric illness, such as depression, schizophrenia; - Autoimmune disease; - Established genetic diseases; - Severe hormonal dysfunctions. This study was approved by Ethic Committee of our Institution and written informed consent from all enrolled subjects was obtained. Participants completed a questionnaire and were physically evaluated by physicians. Cognition was evaluated by the Mini Mental Status Examination (MMSE). MMSE is a brief test of several cognitive abilities with a maximum score of 30 points.

The postmenopausal women were classified into two groups, non-metabolic syndrome (212) and metabolic syndrome (68).

Key eligibility criteria of selection MetS postmenopausal women were according to National Cholesterol Education Program Adult Treatment Panel III [43-44]. The diagnostic criteria of MetS (ATPIII-IDF International Diabetes Federation) included: central obesity, defined as waist circumference of >88 cm and at least two of the following factors: increased serum triglyceride (TG) level: fasting value >150 mg/dl; reduced HDL-C: fasting concentration <50 mg/dl; elevated blood pressure: SBP>130 mmHg

or DBP>85 mmHg; increased fasting plasma glucose (GLU): >100 mg/dl.

2.2 Clinical, Biochemical and Hormonal Measurements

Clinical and anthropometric parameters including blood pressure, weight and height, waist circumference were measured following the standard procedures. Body mass index (BMI) was calculated as weight/height^2 (Kg/m^2). Fasting blood samples were drawn by venipuncture between 7:00 and 9:00 h in order to evaluate the hematological, biochemical, hormonal profile and for extraction of genomic DNA. Hematological and biochemical parameters were measured by standardized methods by chemistry auto-analyzers: Beckman Coulter (hematology), Cobas Mira and Hitachi (biochemistry). Additional, C-reactive protein (CRP), apo B and apoA-I were also measured by chemistry auto-analyzers.

Serum 17OHP, dehydroepiandrosterone(DHEA), dehydroepiandrosterone suphate (DHEAS), A4, estradiol (E2), estrone (E1), progesterone (P), testosterone (T), free testosterone (FT), dihydrotestosterone (DHT), sex hormone binding globulin (SHBG), luteinizing hormone (LH), follicle–stimulating hormone (FSH), PRL, thyroid-stimulating hormone (TSH), growth hormone (hGH), insulin-like growth factor 1 (IGF1), insulin-like growth factor binding protein 1 and 3 (IGFBP1, IGFBP3), insulin, cortisol, parathyroid hormone (PTH), thyroid hormones and bone markers: osteocalcin, cross laps and 25-OH vitamin D (25OHD) were measured using commercial kits supplied by DSL, IBL and DRG Diagnostics with a high precision and sensitivity as previously described [38].

The fasting insulin resistance index (HOMA-IR) using formula: fasting glucose (mmol/liter) X fasting insulin (mIU/liter)/22.5; the apoB/apoA-I and TG/HDL-C ratios as markers of (atherogenic parameter) cardiovascular risk; the cortisol/DHEA ratio as predictive factor in diagnosing aging-related diseases, FSH/LH ratio were calculated. Free androgen index (FAI), free estrogen index (FEI) using the method of Vermeulen (T/E2/ SHBG X 100) was also calculated [45].

2.3 Genotyping

Genomic DNA was prepared from the whole blood using the Maxwell 16 Blood Purification Kit (Promega Inc.). PCR protocols and primers used for genotyping of eNOS-G894T, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase p22(phox) (-930 A/G), CETP TaqIB, ESR1 (PvuII and XbaI) were carried out according to the manufacturer's recommendations, and the products of hydrolysis were separated on agarose gel and visualized with ethidium bromide.

rs1799983 *p*olymorphism of the missense G894T variant of eNOS gene was amplified by PCR with specific primers. The procedure to identify the polymorphism was the same as previously described [1]. The PCR amplified 248-bp fragments were further digested with two restriction enzymes, Ban II and Mbo I, and the products were separated by electrophoresis in 8% nondenaturing polyacrilamide gel, and stained with SYBR Green I.

rs9932581 (-930 A/G) gene polymorphism in the promoter of human p22 phox was amplified by PCR with specific primers [39]. The PCR 650 bp fragments were digested with Bbv I, and the fragments were analyzed by 2% agarose gel electrophoresis.

rs708272 (TaqIB) genotyping: a fragment of 1420-bp was amplified by PCR using specific primers, as previously described [40]. The PCR products were digested with 10U/µl reaction TaqIB restriction enzyme (Promega GmbH, Mannheim, Germany). The digestion product was run on a 2% agarose gel stained with ethydium bromide and visualized under UV light. The CETP B1B1 genotype was identified as two bands of 750bp and 670bp, the B1B2 genotype as 750bp, 670bp and 1420bp, and the B2B2 genotype as the starting undigested 1420bp on the agarose gels.

rs9340799 (XbaI) and ***rs2234693*** *(*PvuII) were evaluated after a genotyping protocol using the AMPLIKIT-ESTR Test-Kit. Both polymorphisms of ESR1gene are in intron 1 and are separated by only 46 base pairs. The PvuII polymorphism is characterized by a T→C transition 397 nucleotides upstream in the intron 1(also known as c.454-497T→C) that obliterates the PvuII restriction site. The T allele has previously been called the p allele, whereas the C allele has been called the P allele, denoting the absence of the PvuII restriction site. The XbaI polymorphism marks an A→G transition 351 nucleotides upstream in intron 1 (also known as c.454- 351A → G). Those with the G allele have an absent XbaI site that was previously called X in the literature, with the A allele denoted by x.

2.4 Statistical Analysis

The measured variables are presented as mean ±SEM. The genotype and allele frequency distributions were compared with the χ2 test and computed Hardy-Weinberg equilibrium for each SNP and group of participants. Variables with a non-symmetrical skewedness distribution were log transformed. The differences in measured variables between groups and among genotypes for each group were tested by with independent Samples T test and by analyses of variance ANOVA post-hoc Bonferroni test. The association analysis between the genotype subgroups were tested using ANOVA or non-parametric Mann-Whitney U and Kruskal– Wallis tests. Binary logistic regression analysis was performed to assess the associations between polymorphism and (components of) MetS. Logistic regression was useful to predict the presence or absence of a characteristic based on values of a set of predictor variables (metabolic/hormonal). It was estimated by a model using stepwise method- forward Wald. Statistical analyses were carried out using SPSS version 15.0 (for Windows) and *P* values less than .05 were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Results

Table 1 summarizes the baseline characteristics with significant differences between our participants with and without MetS. The group with metabolic syndrome had three or more MetS traits and therefore fulfilled the criteria for the MetS. As shown in Table 1, weight, waist circumference, systolic and diastolic blood pressure, WBC, triglycerides, LDL-C, TG/HDL-C ratio, apolipoprotein B, apoB/apoA-I ratio, fasting glucose, insulin, HOMA-IR, uric acid, were higher and HDL-C was lower in MetS group than in control group and therefore fulfilled the criteria for the MetS.

Table 1. Physical and metabolic characteristics were statically significant in MetS group vs Controls

Parameter	Control	MetS	*P*
	Mean (SEM)	Mean (SEM)	
No. of subjects:	212	68	
Age(years)	70.03(0.39)	69.00(0.57)	
BMI((kg/m^2)	26.18(0.39)	27.23(0.96)	
Weight (kg)	66.61 (1.04)	73.02 (1.61)	< .001
Waist circumference (cm)	80.39 (1.63)	96.12 (1.78)	< .001
Systolic BP (mm Hg)	135.89 (1.79)	150.17 (2.56)	< .001
Diastolic BP (mm Hg)	77.60 (0.96)	83.17 (1.26)	< .001
WBC*1000	6.18 (0.11)	6.75 (0.22)	.013
Triglycerides (mg/dL)	116.16 (3.34)	180.44 (9.23)	< .001
HDL- Cholesterol (mg/dL)	62.65 (1.41)	55.47 (3.33)	.022
LDL- Cholesterol (mg/dL)	145.15 (2.77)	167.86(6.51)	< .001
TG/HDL-C ratios	2.07 (0.08)	3.96 (0.33)	< .001
apoB (mg/dL)	105.34 (2.22)	123.34 (4.74)	< .001
apoB/apoA ratios	0.58 (0.01)	0.69 (0.03)	< .001
Glucose (GLU) (mg/dL)	96.75 (1.1.02)	110..32 (2.42)	< .001
Insulin (mIU/L)	9.14 (0.44)	11.49 (0.80)	.009
HOMA-IR	1.06 (0.10)	2.56 (0.64)	< .001
Uric acid (URIC) (mg/dL)	4.79 (0.12)	5.44 (0.24)	< .014
Cortisol (ng/mL)	143.73 (3.46)	128.03 (5.69)	.024
Cortisol/DHEA	22.71 (1.08)	17.02 (17.02)	< .001
E2 (pg/mL)	22.68 (1.56)	37.14 (4.58)	< .001
E2/E1 ratios	0.44 (0.04)	1.07 (0.26)	< .001
SHBG (nmol/L)	60.65 (2.04)	44.45(2.40)	< .001
FSH (mIU/mL)	95.71 (2.35)	78.94 (3.80)	< .001
LH (mIU/mL)	31.75(0.92)	24.60(1.44)	< .001
T3 (ng/mL)	0.91(0.02)	1.06(0.04)	.001
GHBP (pmol/L)	116.06(3.94)	159.76(5.75)	< .001
IGFBP1 (ng/mL)	33.76 (1.30)	27.83 (2.12)	.023
PTH (ng/mL)	62.80 (1.96)	73.31 (4.66)	.020

Key: The data are expressed as mean (SEM); MetS group vs. control group. BP=blood pressure; WBC= white blood cells; HOMA-IR= fasting insulin resistance index; apoB = apolipoprotein B; apoA=apolipoprotein A; URIC= uric acid; DHEA=dehidroepiandrostenedione; E2= estradiol; E1=estrone ; SHBG = sex hormone binding globulin; FSH = follicle stimulating hormone; LH= luteinizing hormone; T3 = triiodothyronine; GHBP = growth hormone-binding protein; IGFBP1 = insulin-like growth factor-binding protein 1; PTH = parathyroid hormone

Additionally, an analysis of endocrine axes showed the significant higher levels of E2, E2/E1 ratio, T3, GHBP, PTH and lower levels of cortisol, SHBG, FSH, LH, IGFBP1, cortisol/DHEA ratio in MetS group as compared with control group.

Taken into account most of biochemical variables are normal range in both groups except glucose, triglyceride, LDL-C, apo B that are in the high borderline range in group with MetS.

The genotypic and allelic distribution of five SNPs were analyzed in MetS group and compared with control group. The results are summarized in tabele 2. Polymorphisms of (930 A/G) p22phox and XbaI ESR1gens appeared to be associated with MetS susceptibility (tab. 2). For rs9932581 (p22phox-A/G), the G allele frequency was significantly lower in MetS than control group and seemed to decrease the risk for MetS (*P*=.022). Comparing the genotype frequencies we detected significant differences in genotype distributions of rs 9340799 (ESR1-XbaI) between MetS and Controls (*P*=.034) and seemed to increase the risk for MetS. However, we didn't observed significant differences in the genotype or allele distributions of three SNPs between MetS and non-MetS.

Significant interactions were detected among gene SNPs that associated with the components of MetS and some endocrine-metabolic variables by binary logistic regression and analyses of variance with post-hoc Bonferroni, T tests and non-parametric (Kruskal–Wallis, Mann-Whitney U) tests (tab.4-7, Annex1-2).

eNOS (G/T) gene polymorphism. Analyses by binary logistic regression (MetS vs Controls) showed a significant interaction of G894T (eNOS) polymorphism with MetS [odds ratio (OR)>2.5; 95% confidence interval (CI)=1.472-4.903] associated with the higher levels of SBP (*P*=.001), TG (*P*<.001), apoB (*P*<.001), URIC (*P*=.006), ASTGOT (*P*=.014) and lower levels HDL-C (*P*=.029) (annex 1.1-2). In MetS group, eNOS (G/T) gene polymorphism significantly associated (*P*<.001) with BP, glucose, HGB, total proteins, cortisol, DHEAS, A4, P, FT and IGFBP3 whose levels are higher (*P*<.05) in TT genotype carriers (annex 2). We also found an association with vitamin D and F/DHEA ratio; levels of vitamin D were higher *(P*<.01) and F/DHEA ratio was lower (*P*=.009) in carriers of T alleles (GT+TT genotype) (annex 2).

Table 2. Chi square test for genotype and allele distributions of SNPs in MetS and control groups

SNP	genotype/allele	Control #(%)	MetS #(%)	λ²	*P*
eNOS-G894T	GG	114(54)	30(44)		
rs1799983	GT	56(26)	22(32)		
	TT	42(20)	16(24)	1.93	.381
	G	284(67)	82(60)		
	T	140(33)	54(40)	2.03	.154
p22phox(A/G)	AA	58(27)	27(40)		
rs9932581	AG	92(44)	28(41)		
	GG	62(29)	13(19)	4.62	.099
	A	208(49)	82(60)		
	G	216(51)	54(40)	5.21	.023
CETP	B1B1	74(36)	27(40)		
TaqIB					
rs708272	B1B2	96(47)	35(51)		
	B2B2	36(17)	6(9)	2.95	.229
	B1	244(59)	89(65)		
	B2	168(41)	47(35)	1.66	.198
ESR1-XbaI(A/G)	AA	82(39)	16(24)		
rs9340799	AG	94(44)	42(62)		
	GG	36(17)	10(15)	6.76	.034
	A	258(61)	74(65)		
	G	166(39)	62(35)	1.77	.184
ESR1-PvuII(T/C)	TT	66(31)	16(24)		
rs2234693	TC	108(51)	38(56)		
	CC	38(18)	14(21)	1.45	.483
	T	240(57)	70(54)		
	C	184(43)	66(46)	1.10	.295

p22phox (A/G) gene polymorphism was in Hardy–Weinberg equilibrium for both control-case groups; tests for association showed that G allele was significantly associated with a decrease in MetS risk with OD=0.634, CI=0.428-0.939, *P*=.022 (Table 3).

In the binary logistic regression analysis (MetS vs Controls), we also detected a protective effect of the 930A>G polymorphism against metabolic syndrome. It was detected an odds ratio between 0.391-0.608 (95% CI=0.222-0.903] associated with higher levels of SBP (*P*=.01), apoB/apoA (*P*<.001), glucose (*P*=.003) (annex 1.3) and the lower levels of DHEAS (*P*=.04), cortisol/DHEA (*P*<.001) (annex 1.4) and LH (*P*<.001), E1(*P*=.04), FSH/LH (*P*=.01) (annex 1.5) In MetS group, the G allele carriers (combined AG and GG genotypes) had lower values of cholesterol (*P*=.019), GH(*P*=.043), IGFBP1(*P*=.001) and osteocalcin (*P*=.024) (Table 4).The GG homozygotes had higher levels of apoB/apoA ratio (Table 4).

CETP TaqIB polymorphism was in Hardy–Weinberg equilibrium for both control-case groups; tests for association didn't show a risk for allele B2, the odds ratio was 0.77 (95% CI=0.516-1.169) and *P*-value .22. The frequency of B2B2 homozygote was more prevalent in controls (17% vs 9%, tab.2). The analysis by binary logistic regression (MetS vs Controls) showed a protective effect of the CETP TaqIB polymorphism against metabolic syndrome, the odds ratio was between 0.001-0.458 (95% CI=0.0001-0.834; *P*=.038) in the presence of SBP (OR=1.375; *P*=.017) (annex 1.6), GLU (OR=1.107; *P*<.001), triglycerides (OR=1.013; *P*=.034) (annex 1.7). It also interacted with MetS associating with the p22phox (A/G) gene polymorphism (OR=0.391, CI=0.222-0.687; *P*<.001), both with OR<1 in the presence of insulin (*P*=.014) and GHBP (*P*<.001), both with OR>1 (annex 1.8). It also associated with the ESR1 (T/C) gene polymorphism whose OR is =8.602 (CI=1.486-49.793, *P*=.016) in the presence of osteocalcin (OD=1.121; *P*=.003) (annex 1.9). In metabolic syndrome the B1B2 heterozygote carriers had higher values of SBP (*P*=.034), glucose (*P*=.012), cholesterol (*P*=.005), HDL-C (*P*=.023), CRP (*P*=.024), GHBP (*P*=.041) and lower levels of TSH (*P*=.032) compared to homozygote carriers (Tab. 5).

ESR1 (XbaI) polymorphism. The tests for association showed that the presence of heterozygote AG indicated a significant risk for MetS (*P*=.011; OR=2.290; CI= 1.198-4.375) (tab.3). The binary logistic regression also indicated a significant association between ESR1 (XbaI) polymorphism and MetS in presence of TG (OR=1.017; *P*<.001), TG/HDL-C ratio (OR=3.614; *P*<.001) (annex 1.10). In MetS group, levels of BMI (*P*=.01), GLU (*P*<.001), HOMA-IR (*P*=.042), 17OHP (*P*=.001), estrone (*P*=.006), T (*P*<.001), GHBP (*P*<.001) were significantly higher and UREA (*P*=.007), URIC (*P*=.024), E2 (*P*=.003), TSH (*P*<.001), vitamin D (*P*<.001) were significantly lower in G allele carriers (AG +GG genotypes)) (Tab. 6).

ESR1 (PvuII) polymorphism. The phenotype of ESR1 PvuII polymorphism in MetS group was partial with that of XbaI polymorphism. ESR1 PvuII polymorphism was in Hardy–Weinberg equilibrium for both control-case groups. Analyses by binary logistic regression (MetS vs Controls) showed significant association of ESR1 PvuII (T/C) polymorphism with MetS (OR between 1.586-8.602) in presence of HDL-C (OR=0.002; *P*<.001), LDL-C (OD=1.016; *P*<.001), TG/HDL-C ratio (OR=2.414; *P*=.003) (annex 1.11), androstenedione (OR=0.355; *P*=.012), SHBG (OR=0.933; *P*<.001), P(OR=7.516; *P*=.022), FAI (OR=0.236; *P*=.027) (annex 1.12) and interacted with CETP TaqIB polymorphism (OR=0.104; *P*=.009) in presence of osteocalcin (OR=1.121; *P*=.003) (annex 1.9). In MetS group, C allele carriers (TC+CC genotypes) had significantly increased levels of glucose (*P*=.003), HOMA (*P*=.03), T (*P*=.03), GHBP (*P*=.002) and decreased levels of ASTGOT (*P*=.001), ASTGPT (*P*<.001), vitamin D (*P*<.001) (Tab. 7).

3.2 Discussion

On the basis of prior evidence for association of eNOS-G894T polymorphism with the metabolic syndrome and published associations with component traits of the metabolic syndrome, we evaluated 5 single nucleotide polymorphisms SNPs for associations with metabolic syndrome and its components in 280 postmenopausal women. In the entire cohort, apparently healthy women, 68 (32.08%) women fulfilled The Adult Treatment Panel III - NCEP criteria for MetS (mean age: 70.03 vs 69.00 years of postmenopausal women without MetS).

Table 3. Tests for association (C.I.: 95% convidence interval) in MetS vs control groups

SNP	Allele freq. difference	Heterozygous	Homozygous	Allele positivity	Armitage's trend test
p22phox	**Risk allele G**				
(A/G)	[A]<->[G]	[AA]<->[AG]	[AA+]<->[GG]	[AA]<->[AG+GG]	common odds ratio
	Odds ratio=0.634	Odds ratio=0.654	Odds ratio=0.450	Odds ratio=0.572	Odds ratio=0.670
rs9932581	C.I.=[0.428-0.939]	C.I.=[0.351-1.218]	C.I.=[0.212-0.956]	C.I.=[0.323-1.013]	
	chi2=5.21	chi2=1.80	chi2=4.43	chi2=3.71	chi2=4.56
	P=.022	*P*=.180	*P*=.035	*P*=.054	*P*=.033
ESR1	Risk allele G				
XbaI(A/G)	[A]<->[G]	[AA]<->[AG]	[AA+]<->[GG]	[AA]<->[AG+GG]	common odds ratio
	Odds ratio=1.302	Odds ratio=2.290	Odds ratio=1.424	Odds ratio=2.050	Odds ratio=1.261
rs9340799	C.I.=[0.882-1.923]	C.I.=[1.198-4.375]	C.I.=[0.589-3.439]	C.I.=[1.098-3.829]	
	chi2=1.77	chi2=6.47	chi2=0.62	chi2=5.19	chi2=1.78
	P=.184 (P)	*P*=.011	*P*=.431	*P*=.023	*P*=.182

Legend: The tests for association are adapted from Sasieni PD (1997). (http://ihg.gsf.de/cgi- bin/hw/hwa1.pl)

Table 4. Physical, biochemical and hormonal characteristics of subjects with MetS by the p22phox (A/G) genotypes

Variables	AA (27)	AG (28)	GG (13)	AG+GG (41)
	Mean (SEM)	Mean (SEM)	Mean (SEM)	Mean (SEM)
BMI	23.96 (2.07)	28.79 (1.02)	31.10 (1.22)*	29.40 (0.83)**
Cholesterol	251.69(5.62)	219.46 (9.42)*	238.83(15.45)	225.58 (8.10)**
apoB/apoA	0.66 (0.06)	0.64 (0.03)	0.88 (0.07)*	0.71 (0.03)
URIC	5.89 (0.41)	4.81 (0.24)*	7.74 (0.37)*	5.29 (0.31)
hGH	2.22 (0.36)	1.41 (0.38)	1.22 (0.34)	1.35 (0.25)*
IGFBP1	36.52 (4.01)	24.14 (2.97)*	17.44 (2.02)**	22.02 (2.17)**
osteocalcin	24.13 (3.58)	15.80 (2.95)	13.67 (1.96)	15.13 (2.10)*

*( )=# genotypes; *=P <.05; ** = P<.01*

Table 5. Physical, biochemical and hormonal characteristics of subjects with MetS by the CETP TaqIB (B1/B2) genotypes

Variables	B1B1 (27)	B1B2 (35)	B2B2 (6)	B1B2+B2B2 (41)
	Mean (SEM)	Mean (SEM)	Mean (SEM)	Mean (SEM)
SBP	143 (2)	156 (4)*	147 (11)	154 (4)**
GLU	102.00 (3.06)	116.88 (3.26)*	115.00 (6.97)*	116.45 (3.3)**
TG	168.08 (10.32)	178.65 (15.09)	262.33 (33.68)*	191.73 (14.73)
Cholesterol	221.54 (9.38)	257.94 (6.97)**	224.00(10.51)	252.85 (6.39)**
HDL-C	46.05(2.06)	64.95(6.02)*	39.83(2.70)	61.19(5.32)*
TG/HDL-C	3.93 (0.34)	3.57 (0.49)	7.01 (1.54)*	4.09 (0.51)
CRP	0.32 (0.07)	0.72 (0.12)*	0.38 (0.11)	0.67 (0.1)**
TSH	4.96 (1.42)	1.81 (0.12)*	1.32 (0.45)*	1.74 (0.12)**
GHBP	146.86 (9.94)	175.42 (7.74)	158.18 (9.72)	171.97 (6.55)*
IGFBP1	27.73 (3.48)	24.26 (2.36)	50.31 (9.61)**	28.17 (2.83)
IGFBP3	167.39 (5.48)	191.73 (7.77)	169.27 (15.75)	188.36 (7.06)*
Cross Laps	0.78 (0.08)	0.53 (0.06)	0.48 (0.18)	0.52 (0.06)**
FSH:LH	4.01 (0.27)	3.45 (0.21)	2.67 (0.09)	3.34 (0.047)*

*( )=# genotypes; *=P <.05; ** = P<.01*

Table 6. Physical, biochemical and hormonal characteristics of subjects with MetS by the ESR1 (Xbal)(A/G) genotypes

Variables	AA(16)	AG (42)	GG(10)	AG+GG (52)
	Mean (SEM)	Mean (SEM)	Mean (SEM)	Mean (SEM)
BMI	22.90 (3.24)	28.50 (0.79)*	29.02 (0.89)*	28.61 (0.64)**
GLU	102.13 (1.76)	108.24 (2.91)	132.20 (7.76)**	112.85 (3.05)**
HOMA-IR	1.18 (0.30)	3.45 (1.00)	1.09 (0.36)	2.99 (0.82)*
UREA	46.55 (6.22)	35.41 (1.25)*	34.57 (2.94)	35.28 (1.13)**
URIC	6.37 (0.6)	5.00 (0.27)*	5.62 (0.39)	5.13 (0.23)*
17OHP	0.46 (0.05)	0.83 (0.10)	0.99 (0.32)	0.86 (0.10)**
F/DHEA	21.58 (2.83)	15.59 (1.14)*	15.76 (1.02)	15.62 (0.94)*
E2	61.00 (14.79)	31.96 (3.81)*	20.72 (7.46)*	29.80 (3.42)**
E1	47.06 (6.86)	87.82 (19.990	170.77 (47.81)*	103.77 (18.92)**
E2/E1	2.80 (0.95)	0.64 (0.11)	0.12 (0.020**	0.54 (0.09)*
T	0.30 (0.02)	0.52 (0.04)**	0.61 (0.10)**	0.54 (0.04)**
TSH	6.69 (2.19)	2.04 (0.19)**	1.55 (0.28)*	1.94 (0.17)**
GHBP	114.38 (5.86)	169.45 (6.94)**	170.46 (10.41)**	169.63 (5.97)**
25OHD	36.12 (5.08)	19.49 1.10)**	23.35 (0.20)**	20.19 (0.95)**

*( )=# genotypes; *=P <.05; ** = P<.01*

Table 7. Physical, biochemical and hormonal characteristics of subjects with MetS by the ESR1 PvuII (T/C) genotypes

Variables	TT(16)	TC (38)	CC(14)	TC+CC(52)
	Mean (SEM)	Mean (SEM)	Mean (SEM)	Mean (SEM)
GLU	99.75 (3.32)	109.63 (2.92)	124.29 (6.47)**	113.58 (2.87)**
HOMA-IR	1.07 (0.32)	3.41 (1.10)	1.99 (0.53)	3.03 (0.82)*
ASTGOT	42.16 (12.86)	17.29 (0.75)**	15.98 (0.74)*	16.92 (0.58)**
ASTGPT	32.36 (7.44)	16.87 (0.84)**	14.28 (0.86)**	16.13 (0.66)**
T	0.36 (0.06)	0.50 (0.04)	0.57 (0.07)*	0.52 (0.03)*
GHBP	122.14 (14.16)	167.41 (6.20)**	169.45 (13.33)*	167.94 (5.67)**
25OHD	36.12 (5.08)	20.04 (1.17)**	20.64 (1.56)**	20.19 (0.95)**

*( )=# genotypes; *=P <.05; ** = P<.01*

All five SNPs were significantly associated with one or more MetS traits ($P \leq .001$ to P=.038) (annex 1). Two of 5 SNPs, p22phox (A/G) and ESR1 (Xbal) showed evidence of significant association with metabolic syndrome except that p22phox (A/G) polymorphism showed a protective effect against metabolic syndrome as summarized in Tables 2&3. Significant associations were detected between SNPs and endocrine-metabolic profile (Tab.4-7; annex 1-2). In addition, our study reported the significant higher serum levels of uric acid, apolipoprotein B,

insulin, HOMA-IR, ratios of TG/HDL-C and apoB/apoA-I in MetS group compared to control group. Hyperuricemia has been associated with several metabolic and cardiovascular conditions, including MetS [46-47]. We found that the higher level of uric acid in MetS group compared to controls were correlated positively with CRP (*P*=.016), E2 (*P*=.001), FAI (*P*=.05), GHBP (*P*=.005), PTH (*P*<.001) and negatively with SHBG (*P*=.034), cortisol (*P*=.001), FT4 (*P*=.02). The increased levels of HOMA-IR and ratio of TG/HDL-C and of the apoB/apoA in MeS group are metabolic conditions for developing atherosclerosis, CVD and type-2 diabetes mellitus.

An analysis of endocrine axes showed significant high serum levels of E2, T3, GHBP, PTH, E2/E1 ratio and low levels of cortisol, SHBG, FSH, LH, IGFBP1, cortisol/DHEA ratio in MetS group as compared with control group (Table.1). The increase of estradiol is amplified by the decrease in gonadotropins (LH and FSH) and SHBG, and the subsequent increase in E2:E1 ratio. The higher level of T3 values was previously reported [20]. The lower levels of SHBG associated with higher levels of E2 in postmenopausal women with metabolic syndrome were also reported by Wienberg ME et al [33]. Unexpectedly, cortisol and gonadotropins values were lower in MetS group than in control group. Despite the fact that the ratio of cortisol/DHEA was significantly decreased no alteration of cortisol/DHEAS ratio (0.375±0.083 vs 0.421±0.032; *P*=0.611) was found. In the Pearson's correlation, cortisol values positively correlated with DHEA (*P*<0.001) and FSH (*P*=0.02). In literature, the reported association of cortisol/DHEAS ratio with metabolic syndrome was observed in Vietnam Experience Study from 584 veterans (age=38.74±2.52yr) [48]. We may speculate that cortisol decline is a consequence of endocrine-metabolic alterations in postmenopausal women older than 60 years with MetS and could contribute at accelerated aging.

We found an association between polymorphism of eNOS Glu298Asp and SBP, TG, apoB, HDL-C and uric acid, some features of the metabolic syndrome. The TT genotype carriers had higher levels of BP and glucose. We also found that the TT genotype carriers had higher levels of DHEAS, A4 and FT. Hyperandrogenism has also been postulated to be a marker for insulin resistance- key feature of the metabolic syndrome and a risk factor for cardiovascular disease [49-51]. Thus, defects in eNOS function may contribute for the development of metabolic syndrome as a risk factor for cardiovascular disease [2-5,52]. The increased prevalence of the metabolic syndrome after menopause may be a result of the metabolic consequences of central fat redistribution which may influence the tendency to develop the metabolic syndrome [25, 30-31,49].

The tests for association with metabolic syndrome showed a risk allele A of p22-phox -930A/G polymorphism that significantly interacted with metabolic syndrome, a significant increase in the A allele frequency was found in MeS subjects. We detected a protective effect against metabolic syndrome of both p22 (phox) 930 A/G and CETP TaqIB polymorphisms in presence of insulin and GHBP using the binary logistic regression. However, the higher levels of BMI and apoB/apoA ratio were detected in GG genotype carriers than in AA genotype individuals in metabolic syndrome. Overall, these findings indicate the susceptibility of p22-phox -930G allele for metabolic syndrome in accordance with some studies that demonstrated a functional role of this polymorphism [39,53]. Although other reports assessing the functional role of this polymorphism in NADPH oxidase activity yielded conflicting results [54]. Although the reasons by which the 930G protectively influenced against the development of MeS were not apparent in the present study, some explanations can be proposed. In this regard, aging is widely acknowledged to be associated with oxidative stress, typically referred to as the free radical theory of aging. In addition, oxidative stress is implicated in age-related disorders such as obesity, hyperglycemia, hyperlipidemia, and hypertension [6,9].

Cholesteryl ester transfer protein plays a key role in human lipid metabolism, and therefore CETP gene polymorphisms may alter susceptibility to MetS. In our study the tests for association of CETP TaqIB polymorphism no indicated a significant association with MetS while the analyses by binary logistic regression (MetS vs Controls) showed an OR<1 in presence TAS, GLU, TG, osteocalcin and ESR1 (T/C) gene polymorphism with OR>1. Also, CETP TaqIB polymorphism associated with MetS (OR<1) in presence of the p22phox (A/G) gene polymorphism whose OR was less than 1 and in presence of insulin and GHBP, both with OR>1. These results sustain that MetS is less likely to occur in carries with CETP TaqIB polymorphism. However it associated with one or more MetS

traits as TAS, GLU, TG. Most studies have been shown that the B2B2 genotype is associated with higher HDL-C levels than the B1B1 genotype [11,13]. Wu Z et al concluded that their results lend support to the concept that increased HDL-C cannot be translated into a reduction in CAD risk [41]. In our MetS group, the levels of HDL-C, TAS, glucose, cholesterol, CRP, free testosterone were higher and TSH, cross laps were lower in the (B1B2) heterozygote carriers. We did not conclude any association of phenotypic features with indices of the metabolic syndrome in B2B2 genotype carriers because the studied group was relatively small.

ESR1 intronic polymorphisms variants, PvuII and XbaI regarding as potential genetic predispositions to metabolic syndrome are controversial [42,55]. In animal models, ESR1 knockout mice have insulin resistance, impaired glucose tolerance, and obesity, indicating that variation in estrogen receptor signaling may have relevant metabolic effects. Studies in postmenopausal women have found associations between estrogen receptor genes (ESR1 or ESR2) and the metabolic syndrome components, particularly obesity and dyslipidemia [14-16,52]. We found that the presence of heterozygote AG showed a significant risk for MetS. The phenotype of ESR1 PvuII polymorphism in MetS group was partly to that of XbaI polymorphism. ESR1 PvuII (T/C) polymorphism associated with MetS in presence of HDL-C, LDL-C, and TG/HDL-C ratio. In MetS group, C allele carriers (TC+CC genotypes) had significantly increased levels of glucose and HOMA-IR. In addition, the PvuII (T/C) variant interacted with CETP-TaqIB. All together these findings sustain the functionality of these polymorphisms in metabolic syndrome.

To the best of our knowledge, the current study is the first to evaluate the interaction of candidate genes between hormonal status and metabolic syndrome in postmenopausal women. An important point is that each of the five SNPs has an association with one or more components of MetS. The defects of eNOS-G894T, p22(phox) (-930 A/G), CETP-TaqIB, ESR1 (PvuII and XbaI) genes may alter susceptibility to MetS, influencing diabetes and CVD risk through effects on components of the metabolic syndrome, specifically increased levels of glucose and HOMA-IR, increased ratios of TG/HDL-C and apoB/apoA.

4. CONCLUSION

Our results sustain an interaction between the studied polymorphisms and their phenotypes in conferring a higher susceptibility to the endocrine-metabolic changes involved in pathogenesis of MetS. The elevated values of TG/HDL-C and apoB/apoA ratios could be risk indicators for calculation cardiovascular risk in of MetS.

ACKNOWLEDGEMENTS

Funding: This work was supported by R&D National Research Programme (CEEX no. 100/2006) of the Ministry of Education and Research.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDIX

Annex 1. Logistic regression variables- groups (MetS vs Control)

1.1. METHOD = FSTEP(WALD) eNOS SBP DBP LgapoA TG apoB LgHDL-C LgInsulin apoB/apoA								
Variables in the Equation		**B**	**S.E.**	**Wald**	**Sig.**	**Exp(B)**	**95.0% C.I.for EXP(B)**	
						OR	**Lower**	**Upper**
Step 5(e)	**eNOS**	**1.03**	**0.29**	**12.85**	**0.000**	**2.795**	**1.593**	**4.903**
	SBP	0.04	0.01	11.83	**0.001**	1.037	1.016	1.059
	TG	0.02	0.00	12.72	**0.000**	1.016	1.007	1.025
	apoB	0.02	0.01	12.56	**0.000**	1.023	1.010	1.036
	LgHDL-C	-3.28	1.50	4.79	**0.029**	0.038	0.002	0.710
	Constant	-6.68	3.22	4.31	**0.038**	0.001		
1.2. METHOD = FSTEP(WALD) eNOS p22phox CETP VSH1h URIC LgASTGOT LgALTGPT HGB								
Step 3(c)	**eNOS**	**0.93**	**0.28**	**11.21**	**0.001**	**2.539**	**1.472**	**4.382**
	URIC	0.41	0.15	7.70	**0.006**	1.511	1.129	2.024
	LgASTGOT	3.13	1.27	6.06	**0.014**	22.799	1.890	274.986
	Constant	-9.25	2.23	17.16	**0.000**	0.000		
1.3. METHOD = FSTEP(WALD) p22phox ESR1(A/G) SBP DBP LgapoA apoB/apoA GLU Cholesterol LgCRP								
Step 4(d)	**p22phox**	**-0.69**	**0.27**	**6.64**	**0.010**	**0.503**	**0.298**	**0.848**
	SBP	0.02	0.01	6.42	**0.011**	1.023	1.005	1.040
	apoB/apoA	3.63	1.01	12.82	**0.000**	37.834	5.179	276.367
	GLU	0.03	0.01	8.84	**0.003**	1.029	1.010	1.049
	Constant	-8.11	1.77	21.08	**0.000**	0.000		
1.4. METHOD = FSTEP(WALD) eNOS p22phox CETP ER1(T/C) ESR1(A/G) F DHEA DHEAS LgF/DHEA								
Step 3(c)	**p22phox**	**-0.50**	**0.20**	**6.08**	**0.014**	**0.608**	**0.410**	**0.903**
	DHEAS	-1.09	0.55	3.99	**0.046**	0.336	0.115	0.980
	Lg(F/DHEA)	-2.97	0.84	12.44	**0.000**	0.051	0.010	0.267
	Constant	4.01	1.34	8.95	**0.003**	54.994		
1.5. METHOD = FSTEP(WALD) eNOS p22phox CETP ESR1(A/G) ESR1(T/C) T FSH LH LgE2 LgP LgE1 LgPRL LgFSH/LH LgE2/E1								
Step 4(d)	**p22phox**	**-0.57**	**0.22**	**6.48**	**0.011**	**0.566**	**0.366**	**0.877**
	LH	-0.06	0.02	15.60	**0.000**	0.940	0.912	0.970
	LgE1	-0.97	0.47	4.22	**0.040**	0.378	0.150	0.956
	Lg(FSH/LH)	-1.82	0.76	5.72	**0.017**	0.162	0.036	0.720
	Constant	5.54	1.65	11.20	**0.001**	253.568		
1.6. METHOD = FSTEP(WALD) CETP SBP DBP BMI TG Cholesterol LDL-C URIC TP apoB LgHDL-C LgASTGOT LgALTGPT LgCRP LgapoA1 LgInsulin LgHOMA-IR apoB/apoA LgTG/HDL-C								
Step 2(b)	**CETP**	**-6.85**	**3.30**	**4.31**	**0.038**	**0.001**	**0.000**	**0.684**
	SBP	0.32	0.13	5.70	**0.017**	1.375	1.059	1.786
	Constant	33.39	14.23	5.50	**0.019**	0.000		

1.7. METHOD = FSTEP(WALD) p22phox CETP ESR1(A/G) ESR1(T/C) GLU TG LDL-C URIC apoB LgInsulin LgHOMA-IR apoB/apoA LgTG/HDL-C Cholesterol								
Step 3(c)	**CETP**	**-2.96**	**0.89**	**11.03**	**0.001**	**0.052**	**0.009**	**0.298**
	GLU	0.10	0.03	15.84	**0.000**	1.107	1.053	1.164
	TG	0.01	0.01	4.51	**0.034**	1.013	1.001	1.024
	Constant	-9.04	2.37	14.56	**0.000**	0.000		
1.8. METHOD = FSTEP(WALD) eNOS p22phox CETP ESR1(A/G) ESR1(T/C) GHBP IGF1 IGFBP1 IGFBP3 LgInsulin LghGH								
Step 4(d)	**CETP**	**-0.78**	**0.31**	**6.53**	**0.011**	**0.458**	**0.252**	**0.834**
	p22phox	**-0.94**	**0.29**	**10.63**	**0.001**	**0.391**	**0.222**	**0.687**
	GHBP	0.02	0.00	26.45	**0.000**	1.021	1.013	1.029
	LgInsulin	1.72	0.86	3.96	**0.046**	5.558	1.027	30.072
	Constant	-2.59	1.05	6.05	**0.014**	0.075		
1.9. METHOD = FSTEP(WALD) eNOS p22phox CETP ESR1(A/G) ESR1(T/C) CrossLaps Osteocalcin 25OHD PTH								
Step 3(c)	**CETP**	**-2.27**	**0.86**	**6.88**	**0.009**	**0.104**	**0.019**	**0.564**
	ESR1(T/C)	**2.15**	**0.90**	**5.77**	**0.016**	**8.602**	**1.486**	**49.793**
	Osteocalcin	0.11	0.04	8.81	**0.003**	1.121	1.039	1.208
	Constant	-5.15	2.06	6.22	**0.013**	0.006		
1.10. METHOD = FSTEP(WALD) ESR1(A/G) apoB/apoA LgTG/HDL-C ESR1(T/C) TG LDL-CLgHDL-C								
Step 3(c)	**ESR1(A/G)**	**0.66**	**0.25**	**6.67**	**0.010**	**1.929**	**1.172**	**3.176**
	TG	0.02	0.00	30.64	**0.000**	1.017	1.011	1.023
	Lg(TG/HDL-C)	1.28	0.34	14.61	**0.000**	3.614	1.870	6.985
	Constant	-3.99	0.67	35.37	**0.000**	0.018		
1.11. METHOD = FSTEP(WALD) ESR1(T/C) apoB/apoA LgTG/HDL-C LDL-C LgHDL-C								
Step 6(e)	**ESR1(T/C)**	**0.46**	**0.23**	**3.95**	**0.047**	**1.586**	**1.006**	**2.500**
	LgHDL-C	-6.44	1.45	19.67	**0.000**	0.002	0.000	0.027
	LDL-C	0.02	0.00	14.97	**0.000**	1.016	1.008	1.025
	Lg(TG/HDL-C)	0.88	0.30	8.62	**0.003**	2.414	1.341	4.347
	Constant	7.13	2.31	9.53	**0.002**	1242.68		
1.12. METHOD = FSTEP(WALD) eNOS p22phox CETP ESR1(A/G) ESR1(T/C) A4 T DHT LH LgE2 LgE1 DHEA DHEAS SHBG FSH Lg17OHP LgP LgFT LgPRL LgFEI LgFAI Lg(E2/E1)								
Step 5(e)	**ESR1(T/C)**	**0.80**	**0.37**	**4.62**	**0.032**	**2.217**	**1.073**	**4.581**
	A4	-1.04	0.41	6.33	**0.012**	0.355	0.159	0.796
	SHBG	-0.07	0.01	24.08	**0.000**	0.933	0.907	0.959
	LgP	2.02	0.88	5.26	**0.022**	7.516	1.341	42.127
	LgFAI	-1.44	0.65	4.87	**0.027**	0.236	0.066	0.851
	Constant	4.88	1.73	7.95	**0.005**	132.207		

Statistics. For each analysis: total cases, selected cases, valid cases. For each categorical variable: parameter coding. For each step variable(s) entered or removed, iteration history, –2 log-likelihood, goodness of fit, Hosmer-Lemeshow goodness-of-fit statistic, model chi-square, improvement chi-square, classification table, correlations between variables, observed groups and predicted probabilities chart, residual chi-square. For each variable in the equation: coefficient (B), standard error of B, Wald statistic, estimated odds ratio (exp(B)), confidence interval for exp(B), log-likelihood if term removed from model.

Annex 2. Physical, biochemical and hormonal characteristics of subjects with MetS by the G894T (eNOS) genotypes

Variables	GG (30)	GT (22)	TT (16)	GT+TT (38)
	Mean (SEM)	Mean (SEM)	Mean (SEM)	Mean (SEM)
SBP	149 (2)	136 (3)*	167 (6)**	150 (4)
DBP	83 (1)	77 (2)	90 (3)*	83 (2)
GLU	106.13 (3.41)	107.00 (3.95)	122.75 (5.08)**	113.63 (3.34)
Hemoglobin	12.73 (0.230	13.23 (0.16)	13.56 (0.12)**	13.38 (0.10)**
Total Protein	7.15 (0.16)	7.33 (0.09)	7.72 (0.08)*	7.48 (0.07)*
Cortisol	109.35 (8.03)	121.20 (8.2)	172.46 (8.48)**	142.78 (7.19)**
DHEAS	0.49 (0.06)	0.59 (0.05)	0.72 (0.07)*	0.64 (0.039)*
A4	0.95 (0.090	1.17 (0.11)	1.57 (0.13)**	1.34 (0.08)**
P	0.2 (0.02)	0.35 (0.05)	0.42 (0.08)**	0.38 (0.04)**
FT	0.41 (0.08)	0.71 (0.10	1.93 (0.51)**	1.23 (0.24)**
IGFBP3	166.87 (7.01)	172.86 (7.59)	208.26 (9.44)**	187.77 (6.51)*
25OHD	16.75 (0.67)	26.75 (3.44)*	23.4 (0.92)	25.26 (195)**
F/DHEA	19.97 (1.71)	13.27 (0.77)**	16.65 (2.33)	14.69 (1.09)**

*( )=# genotypes; *=P <.05; ** = P<.01*

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