Genetic predisposition in patients with hypertension and normal ejection fraction to oxidative stress

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Abstract

The role of oxidative stress (OXS) due to myocardial nitric oxide synthase (NOS) uncoupling related to oxidative depletion of its cofactor tetrahydrobiopterin (BH_4) emerged in the pathogenesis of heart failure with preserved ejection fraction (HFPEF).

We determined the prevalence of 6 single nucleotide polymorphisms (SNPs) of genes encoding enzymes related to OXS, BH_4 metabolism and NOS function in $\geq$60-year-old 94 patients with hypertension and 18 age-matched controls with normal EF. Using echocardiography 56/94(60%) patients with hypertension had left ventricular (LV) diastolic dysfunction (HTDD+ group), 38/94(40%) patients had normal LV diastolic function (HTDD- group). Four SNPs (rs841, rs3783641, rs10483639, rs807267) of guanosine triphosphate cyclohydrolase-1, the rate limiting enzyme in BH_4 synthesis, 1 (rs4880) of manganese superoxide dismutase, and 1 (rs1799983) of endothelial NOS genes were genotyped using real time PCR method and Taqman probes. Protein carbonylation (PC), BH_4 and total biopterin levels were measured from plasma samples. No between-groups difference in minor allele frequency (MAF) of SNPs was found. We calculated a genetic score indicating risk for OXS based on the MAFs of the SNPs. A high genetic risk for OXS was significantly associated with HTDD+ even after adjustment for confounding variables [OR(95%CI):4.79(1.12-20.54); $p=0.035$]. In both patient groups PC ($p<0.05$ for both), plasma BH_4 ($p<0.01$ for both) and in the HTDD+ group total biopterin ($p<0.05$) increased vs. controls. In conclusion, in patients with hypertension and normal EF, a potential precursor of HFPEF, a partly genetically determined increased OXS seems to be associated with the presence of LV diastolic dysfunction.

key words: hypertension, heart failure with preserved ejection fraction, oxidative stress

Abbreviations

BH_2=7,8-dihydrobiopterin

BH_4=tetrahydrobiopterin

BMI=body mass index

BSA=body surface area

EF=ejection fraction

GTPCH-1=guanosine-triphosphate-cyclohydrolase-1

HFPEF=heart failure with preserved ejection fraction

IVRT=isovolumic relaxation time

LV=left ventricular

LVM=left ventricular mass

MAF=minor allele frequency

MnSOD=manganese superoxide dismutase

NO=nitric oxide

NOS=nitric oxide synthase

$O_2^{\cdot-}$=superoxide anion radical

OR=odds ratio

OXS=oxidative stress

PC=protein carbonylation

SNP=single nucleotide polymorphism

SOD=superoxide dismutase

Introduction

Hypertension is the most common underlying cause of heart failure with preserved ejection fraction (HFPEF), which partly or entirely accounts for 78-88% of HFPEF cases[1-3]. The transition of hypertensive heart disease to HFPEF is characterized by progressive left ventricular (LV) hypertrophy and deterioration of LV diastolic and atrial function[4]. Oxidative stress (OXS) mainly due to myocardial nitric oxide synthase (NOS) uncoupling as a consequence of the depletion of the NOS cofactor tetrahydrobiopterin (BH_4) may play a decisive role in this transition process[5-8].

Both essential hypertension and LV diastolic dysfunction, the latter is considered the main pathophysiologic mechanism of HFPEF, are partly determined genetically by a large number of genes, each exerting only a small effect[9,10]. Thirty[11] or 30-60 %[12] of blood pressure variation can also be attributed to genetic influences, and environmental exposures account for the remaining 40-70%.

In this study we sought to investigate gene polymorphisms related to both hypertension or endothelial dysfunction and OXS or BH_4 metabolism in order to assess whether there is a genetic predisposition to OXS in patients with hypertension and normal ejection fraction (EF), and if yes, how it is associated with LV diastolic dysfunction in these patients. To this end, we determined the prevalence of 6 single nucleotide polymorphisms (SNPs) of genes encoding enzymes related to OXS, BH_4 metabolism and NOS function. The prevalence of 4 SNPs (rs841 C>T, rs3783641 A>T, rs10483639 C>G and rs8007267 G>A) of guanosine triphosphate cyclohydrolase-1 (GTPCH-1), the rate limiting enzyme in BH_4 synthesis, 1 (rs4880 T>C) of manganese superoxide dismutase (MnSOD) and 1 (rs1799983 G>T) of endothelial NOS (NOS3) genes were determined.

The studied 4 GTPCH-1 SNPs are strongly linked to each other and later it was referred as the „pain-protective” GTPCH-1 haplotype[13]. The minor alleles of these SNPs are associated with reduced pain sensitivity, mildly increased blood pressure and heart rate due to decreased BH_4 production [and consequential decreased nitric oxide-mediated endothelial function and increased vascular superoxide anion radical ($O_2^{-\cdot}$) production], which mainly manifests in pathophysiological situations when BH_4 production would be normally increased due to upregulation of GTPCH-1, e. g. at inflammatory sites or in injured neurons[13] or in blood vessels exposed to high blood pressure, high cholesterol or other cellular stress factors[13].

Manganese superoxide dismutase (MnSOD) encoded by SOD2 gene is an enzyme catalyzing dismutation of $O_2^{-\cdot}$ to hydrogen peroxide and is an important substituent of cellular enzymatic antioxidant defense against OXS[14]. MnSOD proved to be essential for the survival of aerobic organisms, demonstrated by the extremely short survival of MnSOD knockout mice, which died shortly after birth with dilated cardiomyopathy and neurodegeneration[14]. The extensively investigated rs4880 SNP (T>C change at nucleotide level) causes a substitution of valine (GTT) with alanine (GCT) at codon 16. The alanine variant of MnSOD has an α-helical mitochondrial targeting domain, whereas the valine variant of MnSOD has a β-pleated sheet conformation[14-16]. This conformational difference results in a more efficient transport of alanine variant of MnSOD into mitochondria than the valine variant[14-16], which results in a more efficient protection against OXS. The homozygote Ala/Ala genotype has a 30-40% higher MnSOD activity than its Val/Val counterpart[14-16]. The alanine variant is associated with decreased risk for coronary artery disease, myocardial infarction and atherosclerosis, whereas the Val/Val genotype is an independent genetic risk factor for coronary artery disease and vasospastic angina[15-17].

Endothelial nitric oxide synthase (NOS3=eNOS) synthesizes nitric oxide (NO) from L-arginine, which is a key mediator of endothelial function and reduces OXS by scavenging $O_2^{\cdot-}$. The 894G/T substitution within exon 7 in the rs1799983 G>T SNP of NOS3 gene leads to a 298 Glu/Asp substitution in the mature protein. It has been reported that the 298Asp variant has an enhanced susceptibility to intracellular proteolytic cleavage compared with the 298Glu variant, and has been associated with a lower eNOS activity and reduced generation of NO[18,19]. However, other studies have not confirmed these associations[20]. The minor allele variant of the rs1799983 G>T SNP of NOS3 gene may be associated with endothelial dysfunction and increased risk for coronary artery disease[21-23].

Materials and Methods

Patients

The study was conducted from December 2007 to July 2012 at the 3rd Department of Medicine, Semmelweis University, Budapest. The study complied with the Declaration of Helsinki, and was approved by the Institutional Committee on Human Research. All participants signed an informed consent. We designed to prospectively enroll 100 hypertensive patients with normal LVEF (> 50%) and 40 normotensive, healthy controls ≥ 60 years old over 3 years, but even during an extended period we could enroll only 94 hypertensive patients and 18 age-matched controls. Each patient was followed up for at least one year and 44 patients for 3 years (the average follow-up period was 23.3 ± 12.5 months). Each patient underwent a physical examination, an ECG, a detailed echocardiography, a carotid ultrasound and a chest X-ray at annual follow-up examinations. This study is a part of a multipurpose study conducted in the same patients with the objective to provide new

insights into the pathogenesis of HFPEF by investigating its most common precursor state hypertension with normal EF. We conducted 3 studies: 1) investigating the role of oxidative stress, inflammation, prothrombotic state and neuroendocrine activation in the pathogenesis of HFPEF; 2) investigating the genetic predisposition to oxidative stress, 3) testing the MacIver-Townsend hypothesis explaining the mechanism of normal LVEF despite reduced longitudinal LV systolic function..

Eight patients quit the study, and nine patients fulfilled the exclusion criteria (HFPEF developed in two of them) during follow up. We could not perform genetic analysis in 5 patients [2 control and 3 hypertensive patients (1 with normal LV diastolic function and 2 with LV diastolic dysfunction)] for various reasons, including bad quality of isolated DNA or withdrawn approval for genetic testing. Hypertension was defined by a systolic blood pressure > 140 mmHg and/or a diastolic blood pressure > 90 mmHg, or by antihypertensive pharmacotherapy. Blood pressure values are the average of three readings obtained using standard procedures.

Exclusion criteria included diabetes mellitus, more than a mild degree valvular or congenital heart disease, the presence of electrical pacemakers or implantable cardiac defibrillators, prior cardiovascular surgery, an established history of coronary heart disease, prior or ongoing atrial tachyarrhythmias, prior or manifest heart failure, any malignant or immunological disease, anticoagulant or antioxidant treatment, or conditions associated with acute inflammation or stress.

Measurement of oxidative stress parameters

All measurements were carried out from plasma samples. Protein carbonylation (PC) assays were based on the photometric method of Levine et al.[24]. Plasma tetrahydrobiopterin

(BH_4) level was measured using high performance liquid chromatography based on the method of Fukushima and Nixon[25] and modified by Fekkes and Voskuilen-Kooijman[26]. The BH_4 level was calculated from the difference between total biopterin [BH_4 plus 7,8-dihydrobiopterin (BH_2) plus biopterin] level determined by acid iodine oxidation of BH_2 and BH_4 to biopterin and the alkaline-stable oxidized biopterin level (BH_2 plus biopterin) obtained using alkaline iodine, which oxidizes only BH_2 to biopterin. These methods were described in a more detailed way earlier[27].

Molecular biology methods

The total genomic DNA was isolated from peripheral blood using a QIAamp DNA Blood Mini Kit (QiagenGmbH, Hilden, Germany), DNA Isolation Kit for Mammalian Blood (Roche, Mannheim, Germany; Indianapolis, IN, USA). Genotypes for the rs841, rs3783641, rs10483639, rs8007267, rs4880 and rs1799983 polymorphisms were determined using a predesigned Taqman allelic discrimination assay (C_9866639_10, C_25800745_10, C_30444867_20, C_1545138_10, C_8709053_10 and C_3219460_20 respectively). The assay was carried out according to the manufacturer's instructions (Applied Biosystems, Applied Biosystems Group, 850 Lincoln Center Drive, Foster City, CA) on a 7500 Fast Real Time PCR System (Applied Biosystems).

Calculation of minor allele frequency and genetic score

Minor allele frequency (MAF) was calculated by dividing the total number of minor alleles by the total number of alleles in each group.

We devised a genetic score based on the MAFs of the investigated SNPs indicating the predisposition of patients to OXS. The calculation of the genetic score was based on the total number of minor alleles in each subject. In the case of rs1799983 NOS3 SNP the presence of minor allele predisposes to OXS, therefore the homozygotes of each major allele received a score 0, each minor allele heterozygotes received a score 1 and each minor allele homozygotes received a score 2. In the case of rs4880 MnSOD SNP, as the presence of minor allele is protective against OXS, an inverse score was used: major allele homozygotes received a score 2, minor allele heterozygotes received a score 1 and minor allele homozygotes received a score 0. Since the inheritance of the 4 GTPCH-1 SNPs are strongly linked, and 2 haplotypes are representing 91% of the patients, therefore we calculated a haplo4 score the following way: if the number of GTPCH-1 minor alleles was 0 or 1 the score was 0, if the number of GTPCH-1 minor alleles was $\geq$2, the score was 1. Thus, theoretically the total value of the genetic score could have ranged from 0 to 5. Patients were divided into two groups according to the total value of the genetic score: 1.) patients with low genetic risk for OXS with a total genetic score value of 0 or 1; and 2.) patients with high genetic risk for OXS with a total genetic score value of $\geq$2. This definition of patients with low and high genetic risk for OXS is somewhat arbitrary, however our precalculations using other definitions as well showed that this definition could best demonstrate the between-groups difference in genetic risk for increased OXS.

Echocardiography

Standard echocardiography

Echocardiographic imaging was carried out using a Philips iE33 system (Philips Ultrasound, Bothell, WA, USA). Cardiac dimensions and wall thicknesses were measured from two-dimensionally guided M-mode tracings according to the recommendations of the American Society of Echocardiography[28]. LV mass was computed by the Devereux-modified cube formula[29]. The biplane Simpson method was applied to calculate LV end-diastolic and end-systolic volumes, stroke volume, and LVEF. LV diastolic function was assessed using the combination of transmitral Doppler flow, pulmonary venous flow, isovolumic relaxation time (IVRT) and myocardial tissue Doppler septal early diastolic filling velocity (E'), and was graded according to Nishimura and Tajik[30]: Grade 1=impaired relaxation with normal filling pressure, Grade 1a=impaired relaxation pattern with increased filling pressure, Grade 2=pseudonormalized pattern, Grade 3= restrictive pattern. Transmitral flow was acquired from the apical four-chamber view with the sample volume placed at the level of the tips of mitral leaflets. From these traces E/A ratio, E deceleration time (DT), A wave duration and IVRT were determined. Pulmonary venous flow was acquired from the same view by placing the sample volume within the right upper pulmonary vein. From this trace peak systolic forward flow, diastolic forward flow, atrial reversal flow duration and peak velocity were measured.

Color tissue Doppler imaging

Real time color Doppler myocardial imaging data were obtained in the apical four-chamber view. Mitral annular velocities [peak systolic velocity, peak early diastolic filling velocity (E'), peak late diastolic velocity (A') were recorded from the lateral and septal LV walls. The width of the image sector and the depth of the imaging were adjusted to achieve a frame rate more than 180 frames/s. Pulse repetition frequency was set at the lowest possible

level without aliasing. To ensure an appropriate alignment of the Doppler beam with the myocardial segment of interest an insonation angle not exceeding 20° was maintained.

Statistical analysis

All variables are expressed as mean+SD.

One-way analysis of variance (ANOVA) was used for comparisons of patient characteristics as well as of oxidative stress and LV mass parameters followed by Tukey's multiple comparisons test. The Kruskal–Wallis one-way analysis of variance was performed if Bartlett's test indicated heterogeneity of variances followed by Student's two-tailed t-test with Welch's correction. All tests were two-sided, and the level of significance was set at $p<0.05$. The Hardy-Weinberg equilibrium was assessed and comparisons of the allele frequencies between groups and the deviation of these allele frequencies from Hardy-Weinberg equilibrium were made using the chi-square test. The analysis of association of the calculated genetic score based on MAFs of the investigated SNPs indicating predisposition for OXS with the presence of LV diastolic dysfunction was based on 95%CI for the disease odds ratio calculated according to Bland and Altman 2000[31].

Multivariate logistic regression analysis was carried out with adjustment for age, gender, smoking status, obesity (BMI $\geq$30 kg/m^2), levels of HDL and LDL cholesterol and glomerular filtration rate.

Pairwise linkage statistics were tested with the Arlequin software package[32]. This excluded SNP pairs with independent occurrence. Pairwise linkage disequilibrium (LD) statistics [quantified by Lewontin's coefficient (D') and r^2 values] were tested through the detected linkages in Arlequin output with the standard method[33]. This was compared with the latest data found in the HapMap database for general Caucasian population.

Statistical analysis was performed using GraphPad Prism5 (GraphPad Software Inc., San Diego, CA, USA), STATISTICA (version 11; StatSoft, Inc., Tulsa, Oklahoma, USA) and Statistical Package for the Social Sciences (version 22 for Windows; SPSS, Inc., Chicago, Illinois, USA).

Results

Patient characteristics

Hypertensive patients with (HTDD+) or without LV diastolic dysfunction (HTDD-) and healthy controls had similar gender distribution, height, body weight, body surface area, diastolic blood pressure, heart rate, estimated glomerular filtration rate, HDL-cholesterol and hemoglobin concentrations. Medication was similar in the two hypertensive groups (Table 1). There was no difference in age between the control and the whole hypertensive patient group (66.1±4.4 vs. 69.4±7.7 years, the latter data are not shown in Table 1), however, patients in the HTDD+ group were slightly older than those in the control and HTDD- groups. The body mass index (BMI) was higher and the systolic blood pressure was similarly elevated in both patient groups vs. the control group. The serum creatinine value was higher in the HTDD+ group compared with the control group (Table 1). The LDL-cholesterol was decreased in a borderline significant (p=0.0558) fashion in the HTDD- group and significantly in the HTDD+ group compared with controls, probably due to the fact that greater number of patients received statins in the hypertensive groups. The systolic blood pressure was similarly elevated in both patient groups vs. the control group (Table 1).

LV diastolic dysfunction

At the baseline examination LV diastolic dysfunction was not found in 38/94(40%) of patients with hypertension and normal EF (HTDD- group) and 56/94(60%) patients had mild, Grade 1 (54 patients) or Grade 1a (2 patients) LV diastolic dysfunction (HTDD+ group).

LV mass

LV mass (LVM) increased in the HTDD+ group compared with the control and HTDD- groups, while LVM was similar in the HTDD- and control groups. LVM/body surface area (BSA) increased in both patient groups versus the controls, and in the HTDD+ group versus the HTDD- group. LVM/BMI increased in the HTDD+ group versus the controls and in a borderline significant manner (p=0.063) in the HTDD+ versus the HTDD- group (Table 2)[27].

Association of the investigated individual gene polymorphisms with hypertension and LV diastolic dysfunction

Minor allele frequency distribution

After all 6 SNPs were assessed, we confirmed that all alleles were in Hardy-Weinberg equilibrium. In none of the investigated individual SNPs a significant between-groups difference in MAFs was found (Table 3)

GTPCH-1 Haplotype analysis

Our linkage disequilibrium analysis (these data are not shown) demonstrated a strong linkage among all four tested GTPCH SNPs according to the results published by Doehring A. et al.[13] and to the data of the latest HapMap database.

Combined effect of oxidative stress and BH4 metabolism associated SNPs on hypertension and LV diastolic dysfunction

As it was expected, none of the investigated individual SNPs had significantly different MAF compared with controls and was significantly associated with hypertension and LV diastolic dysfunction. Therefore, we devised a genetic score based on the total number of minor alleles of the investigated SNPs in each subject characterizing predisposition to OXS. The distribution of genetic score in different groups is shown in Table 4 demonstrating the decrease of the percentage of low risk and increase of the percentage of high risk patients for OXS in line with the presence of hypertension and LV diastolic dysfunction. Patients with a high genetic score indicating predisposition to OXS had a significantly increased risk for HTDD+ (presence of LV diastolic dysfunction) [odds ratio=OR (95%CI): 4.05 (1.23-13.40); p=0.023] even after adjustment for age, gender, smoking status, obesity, levels of HDL and LDL cholesterol and glomerular filtration rate (adjusted OR (95% CI): 4.79 (1.12-20.54); p=0.035). Patients with a high genetic score showed only a trend for increased risk for hypertension without LV diastolic dysfunction (Table 5).

Protein carbonylation and plasma BH_4 and total biopterin levels

Compared with the controls protein carbonylation (PC) and plasma BH_4 levels were increased

both in the HTDD- and HTDD+ groups and the plasma total biopterin (=BH_4+BH_2+biopterin) level was increased in the HTDD+ group (Table 2).[27]

Discussion

Main findings

In this study we demonstrated that in patients with hypertension and normal EF there may be a genetic predisposition to OXS, which increases in line with the presence of LV diastolic dysfunction even after adjustment for age, gender, smoking habits, obesity, HDL and LDL-cholesterol levels and glomerular filtration rate in multiple logistic regression analysis. LV mass in itself or indexed to BSA or BMI increased in parallel with the presence of LV diastolic dysfunction in our patients as well. Since the development of LV diastolic dysfunction and hypertrophy are important markers of the transition of hypertensive heart disease to HFPEF[4] our results suggest that the greater number of prooxidant alleles is present in a subject with hypertension, the greater might be the likelihood of the transition of hypertensive heart disease to HFPEF. In fact, we verified the presence of OXS in patients with hypertension and normal EF by the measurement of increased protein carbonylation and increased plasma BH_4 and total biopterin levels[27]. Increased plasma BH_4 and total biopterin might suggest OXS is due to myocardial NOS uncoupling, because there is some evidence that increased plasma BH_4 level is associated with decreased vascular (and probably tissue) BH_4 level[7,8], which causes NOS uncoupling, and in animal models of HFPEF myocardial NOS uncoupling as the most likely source of OXS was elegantly verified[5,6]. To the best of our knowledge so far only the single nucleotide polymorphisms of individual genes encoding enzymes related to OXS were studied in hypertensive patients and this is the first study with

the aim to investigate the genetic predisposition of hypertensive patients to OXS.

The rationale behind the selection of investigated gene polymorphisms

The common feature of all investigated gene polymorphisms in this study was that they have been related to both endothelial dysfunction or hypertension and OXS and the function of enzymes encoded by the genes of the investigated SNPs are connected to each other in the cellular defence against OXS. We hypothesized that the prevalence of minor alleles related to increased OXS and endothelial dysfunction or hypertension will be greater in patients with hypertension without LV diastolic dysfunction (HTDD- group) vs. controls, and even greater in patients with hypertension and LV diastolic dysfunction (HTDD+ group) compared with controls and the HTDD- group. This working hypothesis is also consistent with the assumption that the development of LV diastolic dysfunction is considered an important stage and marker of the transition of hypertensive heart disease to HFPEF, and as a corollary this transition process is at least partly genetically determined. Our results confirmed these hypotheses, because the genetic score, based on the MAFs of the investigated individual SNPs, indicating predisposition to OXS increased in line with the presence of LV diastolic dysfunction.

For any multigenic disorder it is always a problem to quantify the importance of certain genetic variants on the phenotype. Our genetic score is a simplified model for quantitative trait heritance. Our model characterized well the associated phenotype and may be a useful marker in the prediction of progression of LV diastolic dysfunction and transition of hypertensive heart disease to HFPEF.

Limitations

In this study we tested only a few (six) SNPs that might be related to increased OXS, however the regulation of redox metabolism, the production and elimination of free radicals in cells is a far more complicated process, involving much more proteins and enzymes encoded by several genes.

Although we verified a genetic predisposition in patients with hypertension and normal EF to OXS in line with the presence of their LV diastolic dysfunction, the exact pathophysiological mechanisms of this genetic predisposition and whether the increased genetic risk for OXS plays an important role in the transition of hypertensive heart disease to HFPEF remain to be clarified in further future studies.

Statins and other drugs, such as renin-angiotensin system inhibitors and beta blockers, used in the treatment of hypertension might have decreased OXS in patients with hypertension compared to controls due to their antioxidant effect[34,35].

Another significant limitations of this study are the small sample size, especially in the control group, and the lack of a verification cohort, therefore these preliminary results need further verification in a greater patient population with the application of a separate verification cohort.

Conclusions

We verified an increased OXS in hypertensive patients with normal LVEF, a potential precursor condition of HFPEF, which may be partly genetically determined. A high genetic risk of OXS was significantly associated with the presence of LV diastolic dysfunction even after adjustment for confounding variables. The genetic score devised to indicate

predisposition to OXS, which was based on the MAFs of 6 investigated SNPs of genes encoding enzymes related to OXS, BH_4 metabolism and NOS function, together with LV diastolic dysfunction, LVM and atrial dysfunction might be a useful marker of the transition of hypertensive heart disease to HFPEF.

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Table 1.^ Patient characteristics

	Controls (n = 18)	HTDD- (n = 38)	HTDD+ (n= 56)
Age (years)	66.1 ± 4.4	66.1 ± 5.6	71.6 ± 8.1*, ##
Sex (F/M)	12/6	29/9	33/23
Duration of HT (years)	0	11.5 ± 11.6	14.4 ± 12.2
Height (cm)	168.7 ± 8.4	164.3 ± 7.5	164.8 ± 8.6
Weight (kg)	70.1 ± 13.2	74.3 ± 18	80.2 ± 25
BMI (kg/m^2)	24.6 ± 3.7	27.6 ± 5.8*	28 ± 4**, #
BSA (m^2)	1.8 ± 0.2	1.8 ± 0.2	1.8 ± 0.3
Se creatinine (μmol/L)	71.6 ±14.8	70.3 ± 14.8	82.8 ± 25.2#
eGFR (mL/min)	82.6 ± 19.9	88.4 ± 26.6	75.4 ± 27.1
HDL-C (mmol/L)	1.59±0.36	1.55±0.39	1.45±0.37
LDL-C (mmol/L)	3.38±0.72	2.95±0.81	2.79±1.12*
SBP (mmHg)	129.5 ± 16.6	146.5 ± 16.2**	148.9 ± 17.9***
DBP (mmHg)	83.8 ± 9.1	85.9 ± 10.9	88.9 ± 10.8
Heart rate (1/min)	71.1 ± 8.3	74.9 ± 9.3	72.2 ± 8.0
Hemoglobin conc. (g/L)	140.9 ± 12.6	137.2 ± 13.1	138.9 ± 14.8
Medications (number of patients)			
BB	1	22	30
ACEI	0	19	35
ARB	0	9	13
CCB	0	15	27
Diuretics	0	22	35
Aldosterone antagonists	0	0	0
Platelet inhibitors	0	14	24
Statin	3	13	29
PPI	2	10	8

^ Modified table from Ref. 27, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. control; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ vs. HTDD- groups. HT=hypertension, BMI=body mass index, BSA=body surface area, eGFR=estimated glomerular filtration rate, HDL-C=HDL-cholesterol, LDL-C=LDL-cholesterol, SBP=systolic blood pressure, DBP=diastolic blood pressure, BB=beta-adrenergic receptor blocker, ACEI=angiotensin convertase enzyme inhibitor, ARB=angiotensin receptor blocker, CCB=calcium channel antagonist, PPI=proton pump inhibitor

Table 2. Oxidative stress and LV mass parameters

Parameter	Controls (n=18)	HTDD- (n=38)	HTDD+ (n=56)
PC (μmol/g)	0.191±0.131	0.306±0.214*	0.29±0.246*
plasma BH_4 (nmol/mL)	0.917±0.351	1.98±2.1**	2.04±1.99**
total biopterin (nmol/mL)	2.01±0.86	3.05±2.8	2.98±2.46*
LVM (g)	166.2±38	196.9±77.6	226.4±66.2***,#
LVM/BSA (g/m^2)	91.6±14.9	106.56±35.9*	122.7±31***,#
LVM/BMI ($g \times m^2/kg$)	6.779±1.218	7.255±2.608	8.253±2.287**

* p<0.05, ** p<0.01, *** p<0.001 vs. control; # p<0.05, ## p<0.01, ### p<0.001 vs. HTDD- groups.

Table 3. The minor allele frequencies of individual SNPs

Gene	SNP	ref. MAF	control MAF	HTDD- MAF	HTDD+ MAF	p	HW p
GTPCH	rs841	0.23	0.13	0.11	0.16	0.580	0.702
GTPCH	rs8007267	0.48	0.13	0.12	0.14	0.907	0.927
GTPCH	rs3783641	0.25	0.16	0.14	0.15	0.939	0.914
GTPCH	rs10483639	0.23	0.13	0.11	0.16	0.580	0.702
MnSOD	rs4880	0.46	0.69	0.57	0.50	0.161	0.792
NOS3	rs1799983	0.34	0.19	0.27	0.29	0.485	0.727

ref. MAF=The minor allele frequencies reported in the latest HapMap database for general Caucasian population, HW p=The p values indicating whether the MAFs of different SNPs are significantly different from or correspond to the Hardy-Weinberg equilibrium

Table 4. Genetic score distribution in the different groups

	patient groups		
Genetic score	**Controls** n=16	**HTDD-** n=37	**HTDD+** n=54
0	3	3	8
1	8	18	11
low risk for OXS:	11/16 (68.7%)	21/37 (56.8%)	19/54 (35.2%)
2	3	8	21
3	2	5	7
4	0	3	7
5	0	0	0
high risk for OXS:	5/16 (31.3%)	16/37 (43.2%)	35/54 (64.8%)

OXS=oxidative stress

Table 5. Association of genetic risk for oxidative stress with left ventricular diastolic function

	Low risk	High risk	Odds ratio (95% CI)	p value	Adjusted odds ratio(95% CI)*	p value
Normotensive (n=16)	11 (68.7%)	5 (31.3%)	1.0		1.0	
Hypertensive without diastolic dysfunction (n=37)	21 (56.8%)	16 (43.2%)	1.68(0.48-5.80)	0.544	1.66(0.38-7.16)	0.498
Hypertensive with diastolic dysfunction (n=54)	19 (35.2%)	35 (64.8%)	4.05(1.23-13.4)	**0.023**	4.79(1.12-20.54)	**0.035**

* Adjustment was carried out for age, gender, smoking status, obesity (BMI $\geq$30 kg/m^2), levels of HDL and LDL cholesterol and glomerular filtration rate.