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Genetic polymorphisms of endothelial nitric oxide synthase in children with primary hypertension

Polimorfizmy genu śródbłonkowej syntazy tlenku azotu u dzieci z pierwotnym nadciśnieniem tętniczym

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Słowa kluczowe

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Summary

Nitric oxide (NO) is the main substance regulating vascular tone. Endothelial dysfunction and decreased production of NO is a well-known phenomenon in adults with cardiovascular disease and especially when concomitant disorders such as diabetes and atherosclerosis are present. However, there are only few data on NO, polymorphisms of endothelial NO synthase (eNOS) and endogenous inhibitors of eNOS in children with primary hypertension. Children with primary hypertension usually are not exposed to other cardiovascular risk factors such as diabetes, nicotinism and clinically evident atherosclerosis. Thus, children with primary hypertension present the first step of development of cardiovascular disease and allow study of pathogenesis of cardiovascular disease not disturbed by influence of other disorders. We discuss findings from studies evaluating polymorphisms of eNOS and especially of G894T polymorphism and endogenous inhibitors of eNOS in hypertensive children and children with cardiovascular risk factors. G894T polymorphism has been established a functional mutation that is associated with a blunted endothelial-dependent vasodilation and is associated with an increased risk of cardiovascular disease and early vascular changes observed especially in young hypertensive subjects. Although there are no data that eNOS polymorphisms are associated with development of PH, there is evidence that eNOS G894T polymorphism is associated with early subclinical arterial injury in early phase of cardiovascular disease, i.e. in adolescents with PH. However, effects of genetic polymorphisms of eNOS may be modified by behavioral and environmental factors.

Streszczenie

Tlenek azotu jest główną substancją regulującą napięcie mięśni gładkich naczyń. Dysfunkcja śródbłonka i zaburzenie generacji tlenku azotu są stałym zjawiskiem obserwowanym w chorobach układu krążenia, szczególnie powikłanych cukrzycą i/lub miażdżycą. Badania dzieci z nadciśnieniem tętniczym pierwotnym pozwalają na uzyskanie dodatkowych informacji o najwcześniejszych etapach rozwoju choroby sercowo-naczyniowej niepowikłanych wpływem dodatkowych czynników ryzyka, które na ogół są obecne u dorosłych z nadciśnieniem tętniczym. W pracy przedyskutowano znaczenie polimorfizmów syntazy śródbłonkowej tlenku azotu w patogenezie nadciśnienia tętniczego pierwotnego i jego powikłań narządowych u dzieci. Wyniki dotychczasowych badań wskazują, że polimorfizm G894T śródbłonkowej syntazy tlenku azotu jest związany z zaburzeniem zależnej od śródbłonka rozszerzalności naczyń oraz z rozwojem subkliniczych uszkodzeń naczyniowych u dzieci z nadciśnieniem tętniczym pierwotnym. Zjawiska te mogą być modyfikowane przez styl życia oraz czynniki środowiskowe takie, jak dieta oraz aktywność fizyczna.

INTRODUCTION

Primary hypertension (PH) in childhood and adolescence is not a benign disease and causes significant target organ damage (TOD) present in 30-40% of children already at the diagnosis of elevated blood pressure (BP) (1-5). The main intermediate phenotypes of

children with PH are metabolic abnormalities typical of metabolic syndrome, oxidative stress and immune activation. Moreover, these abnormalities are also strictly associated with TOD (6-10). Pathogenesis of PH is multifactorial and it seems that different mechanisms are responsible for elevation of blood pressure and development of TOD. However, these mechanisms are interrelated. For instance, elevation of BP may lead to increase of carotid intima-media thickness (cIMT) and arterial stiffness and increased arterial stiffness causes elevation of BP. Moreover, not all hypertensive patients develop TOD. It suggests that some subjects, and in a case of so common diseases as PH, some part of population, is susceptible to development of TOD and as the consequence, to cardiovascular events (11). Heritability studies suggest that interindividual differences of blood pressure (BP) values are, at least in part, explained by genetic factors (40-60%) (12). However, the interaction of age, gender, ethnicity, diet, used medicines and lifestyle behavior complicates these analyses (13). It is evidenced that the functional impact of genetic polymorphisms on cardiovascular disease is greatest among subjects with lower overall risk (14-16). Since children are relatively free of the common environmental and concomitant clinical factors contributing to cardiovascular disease, the genetic associations exerting theirs effects during the long preclinical phase that begins in childhood, are suspected to be more significant (17, 18).

Human and animal studies point to a number of candidate genes, which may be involved in the development of PH and cardiovascular complications but may also interact with environmental parameters. Because PH is a disease of the arterial tree characterized by increased IMT and arterial stiffening, factors influencing endothelial function are potential modulators of susceptibility to develop PH and TOD. Nitric oxide (NO) has been established as a key signaling molecule in vascular homeostasis. Thus, polymorphisms in the gene that encodes endothelial nitric oxide synthase (eNOS) are of interest because of its potential to affect development of PH and TOD (19).

NITRIC OXIDE AND ENDOTHELIAL NITRIC OXIDE SYNTHASE

The discovery of NO, previously known as endothelium-derived relaxing factor, was one of the most significant biological achievements of the 20th century distinguished by the Nobel Prize in Medicine in 1998. Simple NO molecule is a regulator of many physiological processes. It is synthesized by vascular endothelial cells, it is responsible for vasodilatation and is involved in various processes in the nervous, reproductive and immune systems (20). NO is involved in a wide variety of regulatory mechanisms of the cardiovascular system, including vascular tone (i.e. it is the major mediator of endothelium dependent vasodilatation) and vascular structure (e.g. inhibition of smooth muscle cell proliferation), and cell-cell interactions in blood vessels (e.g. inhibition of platelet adhesion and aggregation, inhibition of monocyte adhesion, cytostatic and cytotoxic properties) (20, 21). In addition to its participation in the regulation of vascular smooth muscle tone, NO directly affects mitochondrial respiration and plays important roles in the development of metabolic syndrome (MS) components, such as insulin resistance, endothelial dysfunction, hypertriglyceridemia and chronic adipose tissue inflammation and is involved in different mitochondrial signaling pathways that control respiration and apoptosis (22, 23).

Moreover, impaired NO bioavailability could also be related to a cellular defect in skeletal muscle tissue, where NO regulates metabolic and contractile processes and also basal, insulin-independent glucose transport (24). Experiments with homozygous eNOS knockout mice have definitively proven the relationship between NO and insulin sensitivity because these mice showed increased blood pressure and insulin resistance (25).

Because of these multiple functions, NO is regarded as an endogenous antiatherosclerotic molecule and tight control of NO production is believed to be critically important for the maintenance of cellular and tissue homeostasis (20, 21). There is hardly a disease not associated with altered NO homeostasis and endothelial dysfunction has become synonymous with reduced biological activity of NO. Thus, endothelial- and NO-dysfunction is a hallmark of not only cardiovascular disease and hypertension but also of obesity, diabetes, malnutrition (26).

There are data indicating on the involvement of eNOS in the pathogenesis of PH and the association of a relative or absolute decrease of eNOS activity with various vascular complications in response to hemodynamic workload (27). There are also data indicating that relative or absolute defect in the production of NO by eNOS or an abundant degradation of NO by enhanced oxidative stress (reactive oxygen species) is associated with various vascular complications in response to hemodynamic workload (27, 28).

NO is an essential molecule, nevertheless, its production is not always beneficial, as an excess or diminished NO production can have detrimental effects. Furthermore, cellular effects of NO may depend not only on its concentration, but also on its site of release and duration of action (29). The discrepant beneficial and detrimental effects that have been ascribed to NO may depend on closely regulated levels of NO in the vessel wall (30, 31). Within endothelial cells that line the lumen of all blood vessels eNOS catalyzes calcium-calmodulin-dependent NO synthesis through the conversion of L-arginine to L-citrulline (32). The normal function of eNOS requires dimerization of the enzyme, the presence of the substrate L-arginine and the essential cofactor (6R)-5,6,7,8-tetrahydro-L-biopterin (BH4), one of the most potent naturally occurring reducing agents. Diminished levels of BH4 or L-arginin have been attributed to the failure of eNOS to form dimers. In mo-

nomeric form eNOS (referred to as eNOS uncoupling) catalyzes the reduction of molecular oxygen to the free radical superoxide ($O2^{-}$) instead of NO. Moreover $O2^{-}$ reacts avidly with NO and forms peroxynitrite ($ONOO^{-}$), a much more powerful oxidant, which in turn also leads to eNOS uncoupling and enzyme dysfunction.

Superoxide is a free radical which rapidly reacts with NO reducing its bioactivity and producing peroxynitrite; a strong oxidant that can nitrosylate cellular proteins and lipoproteins (33-35). Recent evidence suggests that increased superoxide production accounts for a significant proportion of the NO deficit in several animal models of vascular disease, including hypercholesterolemia, hypertension, and heart failure (36). In addition to effects mediated by scavenging NO, superoxide directly stimulates mitogenesis in vascular smooth muscle cells and reduces eNOS expression and activity in endothelial cells (36, 37).

Potential sources of vascular superoxide production include nicotinamide adenine dinucleotide phosphate (NAD(P)H)-dependent oxidases, xanthine oxidase, lipoxygenase, mitochondrial oxidases, and NO synthases (36). NAD(P)H oxidases represent major sources of this reactive oxygen species and have been found upregulated and activated in animal models of hypertension, diabetes, and sedentary lifestyle and in patients with cardiovascular risk factors (38). Peroxynitrite, the direct reaction product of $NO\cdot$ and $O2^{-}$, interacts with lipids, DNA, and proteins via direct oxidative reactions or via indirect, radical-mediated mechanisms. These reactions trigger cellular responses ranging from subtle modulations of cell signaling to overwhelming oxidative injury, committing cells to necrosis or apoptosis (20). It is important to note that particularly $ONOO^{-}$, is able to oxidize BH4 to the $BH3\cdot$ radical (38).

The plasma membrane invaginations that form caveolae are also critical for modulation of eNOS activity. Indeed, it is within caveolae that eNOS attains maximal activity and interacts with CAV-1, a 21-24 kDa protein that coats the cytoplasmic surface of caveolae. In caveolae, eNOS activation is modulated through direct-steric inhibition of calmodulin binding with caveolin (39, 40). Agonist activation (or stimulation by shear stress) increases intracellular calcium and calcium-calmodulin binding, which displaces caveolin and reverses its inhibitory effect on eNOS. In addition to this tonic inhibition, interaction with CAV-1 contributes to eNOS concentration in caveolae. A substantial proportion of active eNOS resides in the peri-Golgi area, proper caveolar localization is critical for eNOS activation and maximal activity (41). Reduced activity of eNOS observed in arterial hypertension, can be caused by increased bonding between eNOS and CAV-1, which inhibits the activity of eNOS. CAV-1 binds eNOS via both the caveolin scaffolding domain and its carboxy-terminal domain. Via this interaction, CAV-1 inhibits eNOS function and NO generation. On the contrary, loss of CAV-1 leads to eNOS hyperactivation and uncontrolled NO overproduction. Excess NO, secondary to the loss of CAV-1, induces mitochondrial dysfunction and aerobic glycolysis, via NO effects on the electron transport system, and interactions of NO with free radicals what generates peroxynitrites (42).

Under normal, basal conditions in blood vessels, NO is steadily produced by eNOS and determines vascular tonus. The activity of eNOS is calcium- and calmodulin-dependent. There are two basic pathways for the stimulation of eNOS, and both of them involve release of calcium ions from subsarcolemmal storage sites. First, shearing forces acting on the vascular endothelium generated by blood flow causes a release of calcium and subsequent eNOS activation. Therefore, an increase in blood flow stimulates NO formation (flow-dependent NO formation). Second, endothelial receptors for a variety of ligands stimulate calcium release and subsequent NO production (receptor-stimulated NO formation). Included are receptors for acetylcholine, bradykinin, substance-P, adenosine, and many others vasoactive substances.

The other factors modulating eNOS and NO effects are endogenous inhibitors of eNOS. Asymmetric dimethyl-L-arginine (ADMA), a product of asymmetric protein methylation is one of the most important. Several studies have shown that ADMA is an independent cardiovascular risk factor (21). Elevated ADMA may inhibit NO synthesis by eNOS (via competition with L-arginine) and could even uncouple the enzyme, which in turn may enhance oxidative stress (43).

Enhanced generation of oxygen free radicals increases ADMA concentration by means of a stimulating effect of oxyLDL on the synthesis of ADMA (it stimulates arginine methyltransferase) on one hand, and by an inhibiting effect of oxygen free radicals on dimethylarginine dimethylaminohydrolase (DDAH), an enzyme that degrades ADMA to citrulline and methylarginine.

In comparison to healthy controls, hypertensive patients, both adults and children, show significantly higher concentration of ADMA, as well an increased production of reactive oxygen species (ROS) (7, 44-47). In children with PH higher ADMA values were found in hypertensive children with MS in comparison to hypertensive children without MS (fig. 1). Similarly, ADMA correlated with markers of insulin resistance (7, 48). In our prospective study of children with newly diagnosed PH, the decrease of ADMA concentrations after 1 year of antihypertensive treatment correlated with a decrease of both TG/HDL ratio and direct markers of insulin resistance such as insulin, HbA1c and HOMA-IR. These relationships, observed also in adult patients, suggest that ADMA is the element that links insulin resistance and the arterio-/atherosclerotic process (47-49).

Summarizing, a decline in NO bioavailability may be caused by decreased expression of the eNOS, a lack of substrate or cofactors for eNOS, alterations of cellular signaling leading to inappropriate eNOS activation, inhibition by endogenous inhibitors of eNOS and finally, accelerated NO degradation by radical oxygen species (28).

ENDOTHELIAL NITRIC OXIDE SYNTHASE POLYMORPHISMS IN CHILDREN WITH PH

The gene encoding eNOS is located on the long arm of chromosome 7 (7q35-36) and contains 26 exons and covers 21 kilobase pairs (50). Several polymorphisms of the eNOS gene have been identified, including single nucleotide polymorphism (SNP) in the promoter region (T786C), a variable tandem repeats in intron 4 and a Glu298Asp SNP in intron 7 (50-53).

More than 15 polymorphisms exist in the eNOS promoter that might influence mRNA transcription and reduce gene expression (54). The -786T/C promoter polymorphism influenced transcriptional activity *in vitro* in a luciferase/reporter assay system and was associated with coronary arterial spasm in Japanese subjects (55). In one study, endothelial cells from subjects with the CC genotype exhibited reduced shear stress induced eNOS mRNA transcription, and vascular rings from such subjects had diminished endothelium dependent vasodilation (56). However, the -786T/C polymorphism has shown inconsistent associations with functional measures, and with clinical disease end points. A recent meta-analysis of studies involving 4882 cases and 9366 controls provided marginal evidence of increased risk among CC subjects (54, 57).

One of the most studied eNOS polymorphisms, G to T transversion at nucleotide 894 of exon 7, produces a glutamic acid to aspartic acid substitution at amino acid 298 (894G→T) and can also alter eNOS enzymatic activity (50). eNOS G894T gene polymorphism has been suggested to be linked to the risk of development of PH and vascular complications, however the results are still debatable (19, 58). It has also become clear that it may contribute singly or in combination with other genes polymorphisms to the development of atherosclerosis (19). The eNOS 894T variant was found to associate with coronary heart disease, carotid atherosclerosis and endothelial dysfunction (13, 17, 19, 40, 59-62). This variant is also associated with enhanced vasoconstrictive response to phenylephrine, hypertensive response to endurance training and development of hypertension (19, 63, 64). However, it seems that both distribution and clinical relevance of eNOS G894T polymorphism is different in different ethnic groups (65, 66). Although the metaanalysis of Niu and Qi indicated that the 894T allele may be associated with an increased risk of hypertension in Asians, this association exhibited no significance in Whites (66). Similarly, interethnic differences in distribution of eNOS genetic variants have been described in other studies including black and white Brazilians and comparing Caucasians, Afro-Americans and Asians (66, 67).

There are only few pediatric studies analyzing associations between eNOS polymorphisms and hypertension, obesity and metabolic syndrome, but until recently, the relation of eNOS G894T gene polymorphism and vascular complications in hypertensive children has not been investigated (68, 69). In our study, we explored associations of eNOS G894T gene polymorphism with TOD markers, oxidative stress, metabolic and inflammatory parameters in an ethnically homogenous group of 126 children with newly diagnosed PH and in 83 healthy children (70). We did not find any difference in prevalence of the T allele among hypertensive children (52.4%) and normotensive children (54.2%). Similarly, we did not find any associations between eNOS G894T polymorphism and blood pressure status nor prevalence of MS (70). Similarly, Miranda et al., did not find any association between G894T polymorphism and MS in obese children and adolescents. However they indicated that the CC genotype for the T786C polymorphism of eNOS is associated with MS (69). Souza-Costa et al. compared the distribution of some eNOS haplotypes (T786C, Intron 4 (4a/4b) and G894T polymorphisms) in normotensive obese and in hypertensive obese children with healthy controls and found that only the 786C-4b-G894 haplotypes constellation was more frequent in hypertensive obese children in comparison to other analyzed groups (68).

In our study we found that hypertensive T allele carriers had greater cIMT and tended to have greater albuminuria in comparison with G allele carriers. Moreover, TT homozygotes presented higher birth weight, lower visceral fat accumulations, lower hsCRP and lower heart rate in ABPM, but significantly higher cIMT and a tendency to greater relative wall thickness (RWT) of left ventricle in comparison to GG homozygotes (tab. 1). Also, in control group T allele carriers tended to have greater cIMT (70).

The correlation between T allele and intima-media thickening was also found in some groups of adults (14, 62). Paradossi et al. found that the TT genotype was a predictor of flow-mediated dilation in young healthy individuals without cardiovascular risk factors (16). Similarly, other studies reported that the 894T allele of the eNOS polymorphism associated with carotid atheroma, and with the presence, extent, and severity of angiographically assessed coronary artery disease (19, 61). Moreover, Czarnecka et al. found higher cIMT values both in hypertensive T allele carriers and also among T allele carriers offspring of hypertensive patients (62). Our finding of association between 894T allele and greater cIMT only in hypertensive children suggests that genetic polymorphism of eNOS gene predisposes to arterial injury only when arterial wall is exposed to higher blood pressure. However, it does not mean that eNOS gene polymorphism is associated with elevated blood pressure.

A study of Antoniades et al. in multivariate analysis showed that the presence of the T allele is an independent predictor of the increase in oxidized LDL during the acute phase of myocardial infarction in young men's (71). This finding suggests that G894T polymorphism affects oxidative stress possibly by affecting the ability of eNOS to maintain sufficient NO levels. However, it is unclear whether the presence of the T allele

results in lower NO production because of eNOS instability or higher superoxide production because of increased eNOS uncoupling under special conditions (71).

Recently, the role of G894T polymorphism in regulation of blood pressure status was evaluated in over 2000 children and adolescents participating in European Youth Heart Study (72). It was found that, TT homozygotes had slightly higher blood pressure values in rest compared to GG carriers. Interestingly, this difference was found only in adolescents (pubertal and post-pubertal subjects) but not in prepubertal children. Moreover, physical activity modified genetic effect, which was most apparent in inactive subjects (72). No association between the G894T variant and BP in 8-10-year olds was observed which may indicate that the eNOS genetic risk of hypertension does not manifest to an appreciable degree before puberty.

Functional consequences of G894T polymorphism of eNOS gene were analyzed in healthy volunteers. It occurred that TT allele carriers, i.e. Asp homozygotes, had decreased vasodilatory response to acetylcholine in the forearm, what indicates blunted endothelial-dependent vasodilation (73). Also the observation of Jiménez-Morales indicated that patients with the TT genotype displayed a lower vascular response (lower increase in postischemic capillary flow) compared with the TG and GG genotypes. Interestingly, this response was ameliorated after an intake of meals rich in high-phenol virgin olive oil (74). Similarly, Leeson et al. found positive relation between n-3 fatty acid level and flow-mediated dilation in 894T carriers but not in G894 homozygotes. Additionally, among men, smoking was associated with lower flow-mediated dilation in T allele carriers but not in GG homozygotes (17).

The molecular mechanism of effects of different eNOS polymorphisms is not clear. Sofowora et al. used a variety of techniques to examine the *in vivo* effects of the G894T polymorphism in healthy volunteers. The TT genotypes were found to affect endogenous NO production (reflected by lower excretion of urinary nitrite/nitrate (75). There are studies not limited to analysis of single polymorphisms but which analyzed effects of different haplotypes of eNOS such as SNP in the promoter region (T786C), a variable tandem repeats in intron 4 and a G894T SNP in intron 7. It occurred that subjects who had haplotype "C-4B-G" had the lowest plasma and whole blood nitrite levels (50, 51). Interestingly, there were marked interethnic differences in distribution of different haplotypes (67).

It was shown that the 894T variant disturbs the catalytic activity of eNOS but the precise biological alteration underlying the high risk of this gene variant is still debated (39). Some studies have pointed that eNOS protein containing an aspartate residue at position 298 are more susceptible to cleavage by proteases, which could results in eNOS dysfunction (76, 77). Other studies questioned the results as an artefact caused by Western blotting preparation (78). It was found recently that eNOS production is dysregulated in subjects carrying the TT compared with those carrying the GG genotype. It is proposed, that this gene variant reduces the interaction of eNOS with caveolin-1 and by the way hinders location of eNOS in caveolae and diminishes shear-dependent eNOS activation (40, 79).

G894T polymorphism may lead to a different response of eNOS to different endothelial stimulation, leading to a reduction in its capacity for NO production under conditions of higher activation. Conditions associated with increased vascular oxidative stress and higher endothelial activation may reduce bioavailability of already decreased NO stores, partly explaining the stronger effect of the eNOS genotype on endothelial function in patients with multiple risk factors for atherosclerosis (17, 80). Therefore, it was hypothesized that the activity of eNOS may be modified by G894T polymorphism only under conditions of increased endothelial cell stimulation (71). Because the 894T variant is associated with decreased NO levels, the risk of endothelial dysfunction and vascular changes, this may mean that young subjects with the 894T genotype are at increased risk and may therefore warrant prophylaxis at an early age.

The modulatory role of statins and virgin olive oil may affect cardiovascular system through its interference with NO generation. Thus, it offers new perspectives for the use of statins and phenol rich olive oil in ameliorating cardiovascular disorders, especially in subjects with down regulated eNOS function, i.e. carriers of TT allele of G894T polymorphism. Moreover, the interaction between the genetic variation in eNOS and blood pressure and endothelial function may be also modified by physical activity. Physical activity may strengthen the production and effect of NO in the regulation of BP through endothelial vasodilatation and could be an effective way of controlling BP and regression of early subclinical arterial injury, especially in adolescents with PH (72, 81).

Concluding, the G894T polymorphism has been established a functional mutation that is associated with a blunted endothelial-dependent vasodilation and is also statistically associated with an increased risk of cardiovascular disease and early vascular changes observed especially in young hypertensive

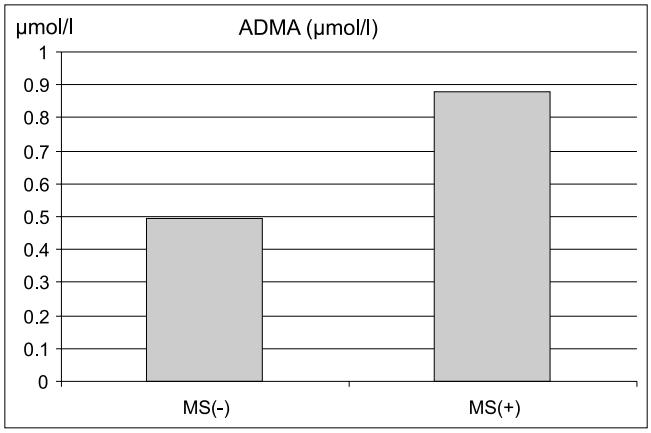

Fig. 1. Comparison of ADMA concentrations (median) between pts with (MS(+)) and without metabolic syndrome (MS(-)) ($p < 0.01$) (7).

subjects (57, 59-61, 70). Although there are no data that eNOS polymorphism are associated with development of PH, there is evidence that eNOS G894T polymorphism is associated with early subclinical arterial injury in early phase of cardiovascular disease, i.e. in adolescents with PH. However, effects of genetic polymorphisms of eNOS may be modified by behavioural and environmental factors.

Table 1. Demographic and clinical data in patients with PH according to eNOS G894T genotype variants (70).

	GG(1)	GT+TT(2)	GT(2a)	TT(2b)	P
n	60	66	53	13	
IMT (mm)	0.43 (0.34-0.52)	0.44 (0.345-0.62)	0.43 (0.345-0.62)	0.465 (0.36-0.57)	1 vs 2 p = 0.01, 1 vs 2a p = 0.05, 1 vs 2b p = 0.02
IMT SDS	0.86 (-1.05-3.12)	1.03 (-1.37-7.2)	1 (-1.37-7.2)	1.49 (-0.35-2.7)	1 vs 2 p = 0.03, 1 vs 2a p = 0.06, 1 vs2b p = 0.03
LVMi (g/m$^{2.7}$)	35.2 ± 8.0	36.9 ± 9.4	36.6 ± 9.4	37.7 ± 9.6	ns
RWT (mm)	0.32 (0.23-0.59)	0.36 (0.23-0.59)	0.35 (0.23-0.59)	0.36 (0.28-0.45)	1 vs 2b p = 0.1

n – number of patients; GG – G894 homozygotes; GT – carriers of G894 and T894 allele; TT – T894 homozygotes; cIMT – carotid intima media thickness; LVMi – left ventricular mass index; SDS – standard deviation score; RWT – relative wall thickness; ns – not significant

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