Eur J Clin Pharmacol (2013) 69:167–177
DOI 10.1007/s00228-012-1326-2

PHARMACOGENETICS

eNOS and *BDKRB2* genotypes affect the antihypertensive responses to enalapril

P. S. Silva · V. Fontana · M. R. Luizon · R. Lacchini · W. A. Silva Jr. · C. Biagi · J. E. Tanus-Santos

Received: 2 April 2012 / Accepted: 24 May 2012 / Published online: 17 June 2012

Abstract
Purpose The antihypertensive effect of angiotensin-converting enzyme inhibitors (ACEi) is attributed partially to increased nitric oxide bioavailability. It is possible that functional polymorphisms in endothelial nitric oxide synthase (*eNOS*) and bradykinin receptor B2 (*BDKRB2*) genes may affect the antihypertensive response to enalapril.
Methods We evaluated 106 hypertensive patients treated only with enalapril for 60 days. The difference between the mean arterial pressure (MAP) before and after the antihypertensive treatment was defined as ΔMAP. If ΔMAP were below or above the median value, the patients were classified as poor responders (PR) or good responders (GR), respectively. *eNOS* genotypes for the $T^{-786}C$, G894T and 4b/4a polymorphisms were determined and haplotype frequencies were estimated by PHASE and Haplo.stats programs. The $C^{-58}T$ and BE1 +9/-9 polymorphisms of *BDKRB2* genes and their haplotypes were determined by DNA sequencing. Robust multifactor dimensionality reduction analysis was used to characterize gene–gene interactions.
Results The TC/CC genotypes and the C allele for the *eNOS* $T^{-786}C$ polymorphism were more frequent in GR than in PR. Furthermore, the TT genotype for the BDKRB2 $C^{-58}T$ polymorphism was more frequent in PR than GR. No other significant differences in genotypes or haplotypes were found. However, we found significant gene–gene interactions: the CC genotype for the *BDKRB2* $C^{-58}T$ polymorphism was associated with response to enalapril depending on *eNOS* $T^{-786}C$ genotypes.
Conclusions These findings suggest that *eNOS* $T^{-786}C$ and *BDKRB2* $C^{-58}T$ polymorphisms may synergically affect the antihypertensive response to enalapril.

Keywords *eNOS* · *BDKRB2* · Polymorphisms · Hypertension · Enalapril

Electronic supplementary material The online version of this article (doi:10.1007/s00228-012-1326-2) contains supplementary material, which is available to authorized users.

P. S. Silva
Department of Pharmacology, State University of Campinas, Campinas, SP, Brazil

V. Fontana · M. R. Luizon · R. Lacchini · J. E. Tanus-Santos (✉)
Department of Pharmacology, Faculty of Medicine of Ribeirao Preto, University of Sao Paulo,
Av. Bandeirantes, 3900,
14049-900 Ribeirao Preto, SP, Brazil
e-mail: tanus@fmrp.usp.br

J. E. Tanus-Santos
e-mail: tanussantos@yahoo.com

W. A. Silva Jr.
Department of Genetics, Faculty of Medicine of Ribeirao Preto, Ribeirao Preto, SP, Brazil

C. Biagi
Santa Casa of Araçatuba,
Araçatuba, SP, Brazil

Introduction

Hypertension is a multifactorial disease that represents one of the most important causes of morbidity and mortality worldwide [1]. One of the most used antihypertensive treatments is the angiotensin-converting enzyme (ACE) inhibitor class. The main mechanisms of action for these drugs include decreased formation of the vasoconstrictor angiotensin (Ang) II, and increased levels of the vasodilator bradykinin. The reduction of blood pressure by this class of drugs has an alternative mechanism involving nitric oxide (NO) vasodilation [2–4]. This secondary mechanism seems

to result from increased endothelial nitric oxide synthase (*eNOS*) protein expression and activity [5–8]. However, as it was shown that bradykinin receptor B2 (*BDKRB2*) inhibitors impair ACE inhibitors NO-dependent effects, the increase in NO availability seems to be partially mediated by BDKRB2 activation [5, 7, 9]. Therefore, it is possible that variations in genes encoding *eNOS* and *BDKRB2* could affect the antihypertensive response to ACE inhibitors.

Three *eNOS* polymorphisms have been widely studied: a $T^{-786}C$ substitution in the promoter region (rs2070744), a variable number of tandem repeats (VNTR) in intron 4 (the allele 4b has five 27-bp tandem repeats while the allele 4a has four repeats) and $G^{894}T$ substitution in exon 7 (rs1799983) resulting in a Glu to Asp substitution at the 298 position in eNOS protein. These polymorphisms have been shown to affect NO production [10–14] and are associated with endogenous NO markers levels in humans [15–17], and with altered risk for hypertension [18–21] and other cardiovascular diseases (CD) [22–25].

Two *BDKRB2* polymorphisms are commonly studied: a $C^{-58}T$ substitution in the promoter region (rs1799722), and an insertion-deletion in the noncoding exon 1 BE1 +9/-9 (the allele +9 has three 9-bp tandem repeats while the allele –9 has two repeats) [26, 27]. These polymorphisms have been associated with modifications in the transcription rate of the *BDKRB2* gene [26] and were associated with altered risk for developing CD [28–30]. Although it was shown that *eNOS* and *BDKRB2* gene polymorphisms may affect the responses to some drugs that increase NO bioavailability [31–35], no previous studies have examined if these polymorphisms modulate the antihypertensive response to ACE inhibitors.

In the present study, our main objective was to assess whether *eNOS* and *BDKRB2* gene polymorphisms could affect the responses to enalapril in hypertensive patients. In addition, we have also evaluated the combined effect of polymorphisms within genes (in haplotypes) and between genes in the response to enalapril.

Methods

Subjects and study design

This study was approved by the Institutional Review Board at the Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Brazil, and each subject provided written informed consent. The present work was carried out in accordance with the ethics standards of the Helsinki Declaration. We recruited 106 subjects diagnosed with mild to moderate hypertension from Cardiology Division of the Araçatuba Health Center (Araçatuba, SP, Brazil). All patients were never-treated or underwent a wash-out period for at least 2 weeks. Patients provided complete health history and underwent physical examination and laboratory analysis. Subjects with evidence of severe or secondary hypertension, other concomitant cardiovascular diseases, or respiratory, hepatic, renal, or hematological dysfunction were excluded. Included patients were treated with enalapril, receiving a dose of 10 mg/day (n=48) or 20 mg/day (n=58) for 60 days. A semi-automatic blood pressure monitor (OMRON® - HEM-433 INT, Bannockburn, Illinois, USA) was used for blood pressure measurements before and after the use of enalapril. Mean arterial pressure (MAP) was calculated using the averages of three different systolic and diastolic blood pressure measurements, with intervals of 1 minute between each other. The formula used to calculate MAP was MAP=(SBP+2*DBP)/3. The responsiveness to enalapril was assessed by the ΔMAP obtained from difference between the MAP before and after treatment with enalapril. According to the response to enalapril, patients were classified as poor responders (PR) or good responders (GR) when their ΔMAP were below or above the median value of the ΔMAP distribution, respectively. After blood pressure measurements, venous blood samples were collected and genomic DNA was extracted by salting-out method and stored at -20 °C until analysis.

Genotyping

Genotypes for the *eNOS* $T^{-786}C$ (rs2070744) and Glu298Asp (rs1799983) polymorphisms were determined by Taqman Allele Discrimination assay using real-time polymerase chain reaction (PCR). Primers and probes for the $T^{-786}C$ polymorphism were customized as follows: forward primer 5′-ACCAGGGCATCAAGCTCTTC-3′, reverse primer 5′-GCAGGTCAGCAGAGAGACTAG-3′ and probes 5′- CAGGGTCAGCC[G/A]GCCA-3′. Primers and probes for the Glu298Asp polymorphism were designed by Applied Biosystems (assay ID: C_3219460-20). Fluorescence was measured on a Chromo 4 Detector (Bio-Rad Laboratories, USA). Genotypes for the *eNOS* VNTR polymorphism were determined by PCR and fragment separation by electrophoresis in 8 % polyacrylamide gels as previously described [32, 36].

The *BDKRB2* $C^{-58}T$ (rs1799722) and BE1 +9/-9 polymorphisms were determined using PCR amplification followed by DNA sequencing. The forward primer 5′-AACGCCCACTGTTTACATCC-3′ and reverse primer 5′-ACGACCACAGGGAAACTTCT -3′ encompassed the polymorphic region as described earlier [37].

Statistical analysis

The clinical and laboratory characteristics of the studied groups were compared by unpaired t test (parametric data),

by Mann Whitney test (non-parametric data), by chi-square test or Fisher's test (categorical variables), where appropriate. Differences in allele and genotypes distributions and deviation from the Hardy-Weinberg equilibrium were tested by chi squared test. A p value <0.05 was considered statistically significant. Given the sample size of this study, we obtained a statistical power of 80 % with an alpha of 0.05, calculated using the PGA Matlab software [38] to detect an odds ratio of 2.5.

eNOS haplotypes were estimated using the program PHASE (version 2.1, http://www.stat.washington.edu/stephens/software.html). To further confirm these haplotype estimates, we have also used the Haplo.stats software (version 1.4.4, http://cran.r-project.org/web/packages/haplo.stats/index.html), which computes maximum likelihood estimates of haplotype probabilities. The possible haplotypes including genetic variants of the three polymorphisms studied here in the *eNOS* gene (T^{-786} C, intron 4 and Glu298Asp) were H1 (T 4b Glu), H2 (T 4a Glu), H3 (T 4b Asp), H4 (T 4a Asp), H5 (C 4b Glu), H6 (C 4a Glu), H7 (C 4b Asp), H8 (C 4a Asp). Only the haplotypes with observed frequencies >2 % were included in subsequent analysis. Additionally, *BDKRB2* haplotypes including two polymorphisms (C^{-58}T and BE1 +9/-9) were determined by DNA sequencing: H1 (C -9), H2 (T -9), H3 (C +9) and H4 (T +9). As the BE1 +9/-9 polymorphism creates a frame shift in the sequencing results, it was possible to exactly determine which allele of the C^{-58}T polymorphism was linked to which allele of the BE1 +9/-9 polymorphism.

Differences in haplotype frequencies among groups were further tested using chi squared test, considering P<0.00833 or P<0.0125 as statistically significant (after Bonferroni's correction: 0.05/number of observed haplotypes) for *eNOS* or *BDKRB2* haplotypes, respectively.

Multifactor dimensionality reduction (MDR) analysis evaluate all possible combinations of genotypes for their ability to classify them into poor and good responder groups through cross-validation (CV) steps and permutation testing [39, 40]. Here we used the Robust MDR (RMDR; http://www.epistasis.org/ [41]) approach to characterize the interaction models among all five polymorphisms studied with a threshold α=0.05. We considered the model that had the maximum testing score and CV consistency (CVC) as the best interaction model. Permutation testing was performed to determine the statistical significance of the best model [40].

Results

Clinical and laboratory characteristics of the 106 hypertensive patients studied are shown in Table 1. As expected, both systolic and diastolic blood pressure was lower after treatment with enalapril (p<0.001). To evaluate if *eNOS* and *BDKRB2* polymorphisms/haplotypes could affect the antihypertensive response to enalapril, these patients were grouped according to responsiveness to antihypertensive therapy: poor responders (PR) or good responders (GR) (see Methods section for details).

There were no differences in sex, ethnicity, age, body mass index or any laboratory parameters between PR and GR groups (Table 1; p>0.05). Additionally, blood pressure was lower after treatment with enalapril in both PR and GR groups (p<0.001). However, baseline systolic and diastolic blood pressure and heart rate was higher in GR (p<0.05).

The genotypes distributions for all *eNOS* and *BDKRB2* polymorphisms did not deviate from Hardy-Weinberg equilibrium (P>0.05). Figure 1 shows the results of a single-locus and haplotype analysis for eNOS gene. Interestingly, good responders showed higher frequencies of the TC and CC genotypes, and of the C allele for the T^{-786} C polymorphism than PR (all P<0.05; and TT *vs*. TC, P=0.0005, Odds ratio (OR)=0.35, 95 % confidence interval (CI)=0.19-0.63; and TT *vs*. CC, P=0.0424, Odds ratio=0.38, 95 % CI=0.14-0.99).

No other significant differences in genotypes/alleles were found with respect to the 4b/4a polymorphism in intron 4 or Glu298Asp in exon 7 (all p>0.05). Additionally, we found that the H7 haplotype (C 4b Asp) is more frequent in GR than in PR patients (P=0.0272; Fig. 1), however this result did not resist to the Bonferroni's correction for multiple comparisons.

The results for *BDKRB2* polymorphisms showed higher frequencies of the TT genotype for the C^{-58} T polymorphism in PR compared with GR (all P<0.05, Fig. 2; TT vs. CT, P=0.0106, OR=3.06, 95 % CI=1.27-7.35; and TT vs. CT+CC, P=0.0175, OR=2.69, 95 % CI=1.16.-6.21).

However, no significant differences were seen in the genotype/allele frequencies of BE 1 +9/-9 polymorphisms between groups. We have also not found significant differences in *BDKRB2* haplotype distribution when poor responders and good responders were compared (P>0.05).

When poor and good responders were stratified according to the dose of enalapril used (Table 2 and 3), we found similar results compared to those shown above. Interestingly, despite the reduction in the sample size in each group, we found significant differences in genotype distributions for the *eNOS* T^{-786} C polymorphism (Table 2; P<0.05) and for the *BDKRB2* C^{-58} T polymorphism (Table 3; P<0.05) between poor and good responders.

When the patients were grouped according to eNOS haplotypes or *BDKRB2* haplotypes, we found no significant differences in blood pressure responses to enalapril (Supplementary Fig. 1; P>0.05).

We have also evaluated gene-gene interactions among all the *eNOS* and *BDKRB2* polymorphisms studied. We found a

Table 1 Clinical and laboratory characteristics of hypertensive patients classified as poor or good responders to enalapril

	All	Poor responders	Good responders
N	106	53	53
Sex (% male)	68	75	60
Ethnicity (% non-Black)	85	83	86
Age (years)	47±12	47±13	48±11
BMI (Kg/m^2)	29.4±5.4	30.0±6.0	28.8±4.7
Total cholesterol (mg/dL)	206±43	200±38	211±48
LDL cholesterol (mg/dL)	129±38	123±24	135±48
HDL cholesterol (mg/dL)	46±14	47±19	44±8
Triglycerides (mg/dL)	152±72	150±69	154±76
Glucose (mg/dL)	91±13	91±16	91±10
Creatinine (mg/dL)	0.91±0.14	0.89±0.11	0.92±0.17
Potassium (mEq/L)	4.2±0.3	4.2±0.3	4.2±0.4
SBP (mm Hg)			
Baseline	149±9	146±8	152±10$^{\#}$
After enalapril treatment	129±9*	133±8*	125±9*$^{\#}$
DBP (mm Hg)			
Baseline	93±9	89±8	97±8$^{\#}$
After enalapril treatment	80±7*	82±8*	78±6*$^{\#}$
HR (beats/min)			
Baseline	78±12	81±12	75±11$^{\#}$
After enalapril treatment	77±11	78±12	77±9

BMI = body mass index; HDL = High-density lipoprotein; LDL = Low-density lipoprotein; SBP = systolic blood pressure; DBP = diastolic blood pressure; HR = heart rate

Values are the mean ± S.D. * P< 0.001 *vs.* baseline; $^{\#}$ P<0.05 *vs.* poor responders

significant interaction between the *eNOS* T^{-786} C and *BDKRB2* C^{-58} T polymorphisms when compared poor and good responders (p=0.045; Table 4).

The specific combinations of genotypes classified as better or worse response to enalapril are depicted in Fig. 3. The combination of the CC genotype for the *BDKRB2* C^{-58} T polymorphism with the TC genotype for the *eNOS* T^{-786} C polymorphism was more frequent in patients that exhibited better response to enalapril treatment. Conversely, the combination of the CC genotype for the *BDKRB2* C^{-58} T polymorphism with the TT genotype for the *eNOS* T^{-786} C polymorphism was more frequent in the patients that exhibited worse response (Fig. 3).

When poor and good responders were stratified according to the dose of enalapril used, or when dose was treated as an independent variable in MDR analysis, we found similar, but not significant, results compared to those shown above (data not shown). Probably, this is due to the reduction in the sample size in each group in the gene-gene interaction analysis.

Discussion

To our knowledge, this is the first study to investigate the association between *eNOS* and *BDKRB2* gene polymorphisms and antihypertensive responses to enalapril. The main findings of this study were that the C allele for *eNOS* T^{-786} C polymorphism is associated with better antihypertensive response to enalapril, whereas the TT genotype for *BDKRB2* C^{-58} T polymorphism is associated with worse antihypertensive response to enalapril in hypertensive patients. In addition, gene–gene interaction analysis showed that patients with CC genotype for the *BDKRB2* C^{-58} T polymorphism were associated with poor or good response depending on the genotype for the *eNOS* T^{-786} C polymorphism.

Previous *in vitro* and clinical studies showed that the *eNOS* T^{-786} C polymorphism can modify the responses to cardiovascular drugs [32, 33, 42–45]. Endothelial cells with CC genotypes treated with statins showed higher *eNOS* mRNA level than cells with the TT genotype [42]. This effect was attributed to increased transcriptional activity of the *eNOS* gene and mRNA stability, and to decreased expression of the transcriptional repressor factor (RPA-1) in cells carrying the C allele. Consistently, results from a clinical study suggested that individuals with CC genotypes may have increased benefits by atorvastatin treatment as they had increased NO bioavailability and attenuated oxidative stress [33]. Besides that, the anti-inflammatory effects of atorvastatin are modulated by this polymorphism [44]. While treatment with statins reduced the circulating

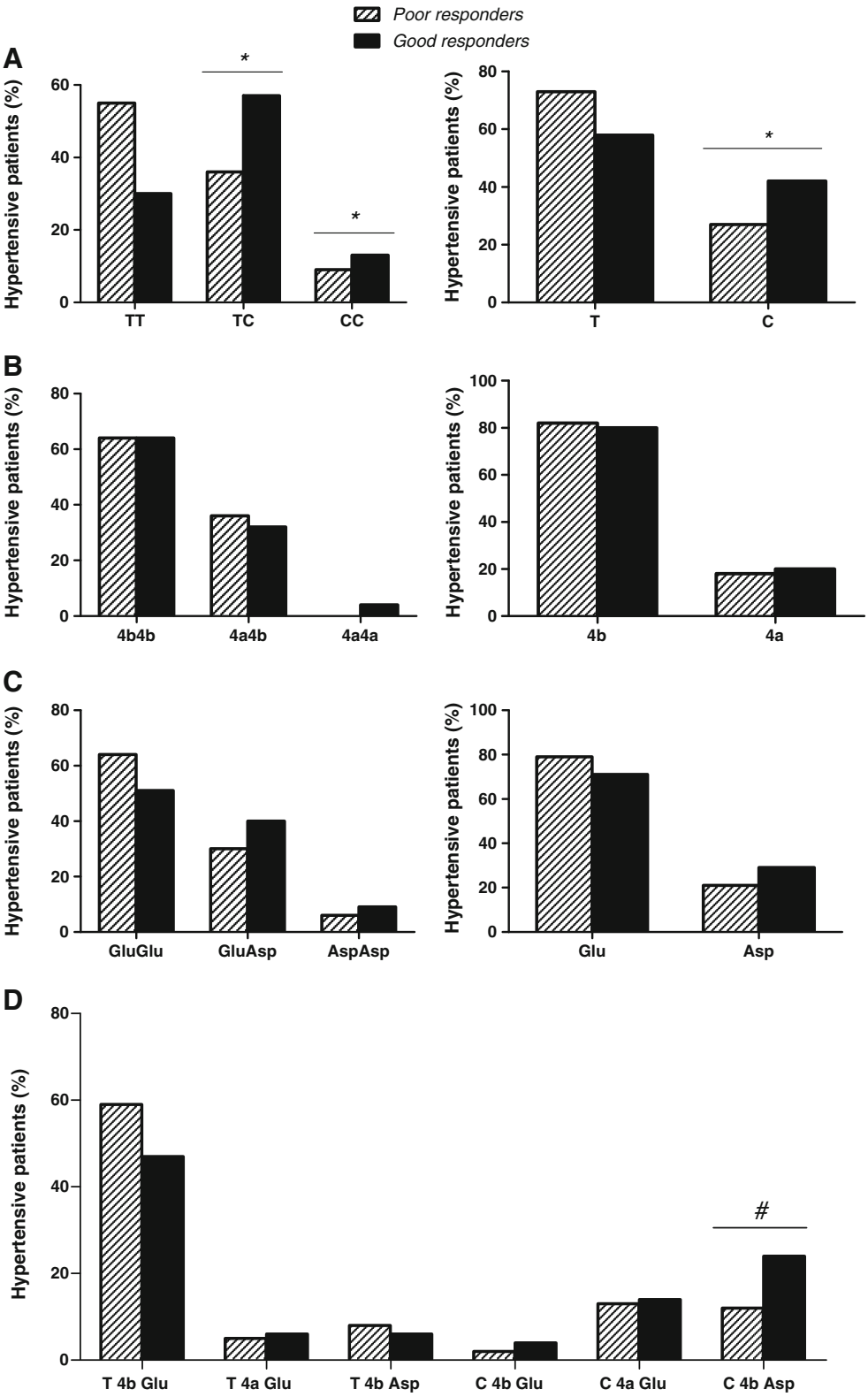

Fig. 1 Genotype, allele and haplotype frequencies distributions (%) for endothelial nitric oxide synthase (*eNOS*) gene polymorphisms in hypertensive patients classified as poor responders (n=53) or good responders (n=53) to enalapril. Panel A: $T^{-786}C$ polymorphism; Panel B: 4b/4a polymorphism; Panel C: Glu298Asp and Panel D: *eNOS* haplotypes. *P<0.05 *vs*. reference genotypes/alleles. #P <0.05 *vs*. all other haplotypes, not statistically significant after Bonferroni's correction for multiple comparisons

concentrations of CD40L, VCAM-1, P-selectin and matrix metalloproteinase-9 in individuals with the CC genotype, no effects were found in these pro-inflammatory mediators in subjects with the TT genotype. Recent findings have suggested that *eNOS* polymorphisms may also modulate the response to other drugs, such as angiotensin receptor blockers (ARBs) [43]. Olmesartan and the other ARBs produced higher NO release from cells homozygous for C

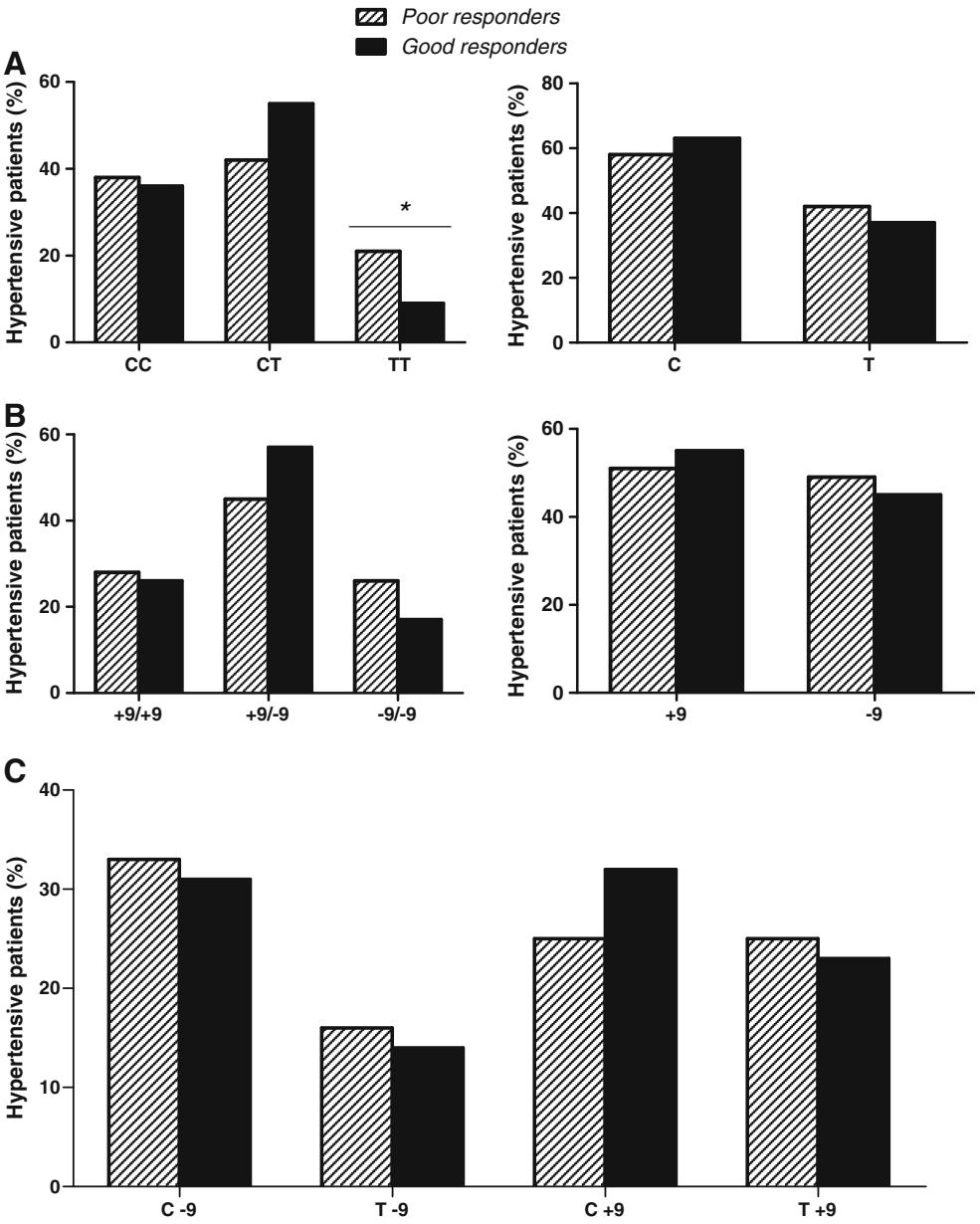

Fig. 2 Genotype, allele and haplotype frequencies distributions (%) for bradykinin receptor B2 (*BDKRB2*) gene polymorphisms in hypertensive patients classified as poor responders (n=53) or good responders (n=53) to enalapril. Panel A: $C^{-58}T$ polymorphism; Panel B: BE1 +9/-9 polymorphism and Panel C: B2 receptor haplotypes. *P<0.05 *vs.* CT genotype

allele endothelial cells compared to cells heterozygous for $T^{-786}C$ polymorphism. Additionally, the response to phosphodiesterase type 5 (PDE-5) inhibitors was modified by $T^{-786}C$ polymorphism in the *eNOS* gene [32]. The authors found that C allele apparently improves the responses to sildenafil in postoperative erectile dysfunction patients, as compared to the T allele. Taken together, these consistent findings provide strong evidence supporting the idea that treatment with statins, ARBs, PDE-5 inhibitors and now, enalapril, may be more beneficial in subjects with the C allele.

Interestingly, GR exhibited higher baseline systolic and diastolic blood pressure than PR. These results are in agreement with previous studies, which also showed that individuals carrying the variant C for the same polymorphism had increased risk of developing cardiovascular diseases, including hypertension [18, 19]. The C allele in the promoter region of the *eNOS* gene is associated with a 50 % decrease of promoter activity [12]. This variant was also associated with decreased NO release by platelets from healthy subjects [46] and lower plasma concentrations of NO metabolites in pre-hypertensive individuals [47].

Conversely, we did not find other significant differences in genotype or allele frequencies with respect to both 4b/4a and Glu298Asp *eNOS* polymorphisms between poor and good responders. Whereas eNOS haplotypes had been

Table 2 Genotype, allele and haplotype frequencies for *eNOS* gene in hypertensive patients classified as poor or good responders to enalapril 10 or 20 mg/day

		Enalapril 10 mg				Enalapril 20 mg			
		PR (n=24)	GR (n=24)	*P*-value	OD (CI)	PR (n=29)	GR (n=29)	*P*-value	OD (CI)
$T^{-786}C$	TT	50(12)	33(8)	-	1.00 (reference)	59(17)	28(8)	-	1.00 (reference)
	TC	42(10)	58(14)	0.014	0.48 (0.26-0.86)	31(9)	55(16)	<0.001	0.27 (0.14-0.50)
	CC	8(2)	8(2)	NS	0.66 (0.23-1.93)	10(3)	17(5)	0.004	0.28 (0.11-0.69)
	X^2-test	6.04		0.049		19.56		<0.001	
	T	71(34)	63(30)	-	1.00 (reference)	74(43)	55(32)	-	1.00 (reference)
	C	29(14)	38(18)	NS	0.67 (0.37-1.20)	26(15)	45(26)	0.005	0.43 (0.24-0.78)
Intron 4	4b4b	67(16)	71(17)	-	1.00 (reference)	62(18)	59(17)	-	1.00 (reference)
	4b4a	33(8)	25(6)	NS	1.40 (0.75-2.60)	38(11)	38(11)	NS	0.95 (0.54-1.69)
	4a4a	0(0)	4(1)	NS	0.12 (0.01-2.23)	0(0)	3(1)	NS	0.14 (0.01-2.70)
	X^2-test	5.21		NS		3.07		NS	
	4b	83(40)	83(40)	-	1.00 (reference)	81(47)	78 (45)	-	1.00 (reference)
	4a	17(8)	17(8)	NS	1.00 (0.48-2.09)	19(11)	22 (13)	NS	0.83 (0.42-1.66)
Exon 7	GluGlu	67(16)	54(13)	-	1.00 (reference)	62(18)	48(14)	-	1.00 (reference)
	GluAsp	29(7)	38(9)	NS	0.62 (0.34-1.12)	31(9)	41(12)	NS	0.59 (0.32-1.07)
	AspAsp	4(1)	8(2)	NS	0.40 (0.12-1.41)	7(2)	10(3)	NS	0.54 (0.19-1.53)
	X^2-test	3.93		NS		3.69		NS	
	Glu	81(39)	73(35)	-	1.00 (reference)	78(45)	69 (40)	-	1.00 (reference)
	Asp	19(9)	27(13)	NS	0.63 (0.33-1.24)	22(13)	31 (18)	NS	0.63 (0.33-1.19)
Haplotypes									
	T 4b Glu	60(29)	52(25)	NS	1.39 (0.79-2.43)	59(34)	43(25)	NS	1.91 (1.09-3.37)
	T 4a Glu	2(1)	6(3)	NS	0.32 (0.06-1.63)	7(4)	5(3)	NS	1.43 (0.44-4.67)
	T 4b Asp	8(4)	4(2)	NS	2.09 (0.61-7.17)	9(5)	7(4)	NS	1.31 (0.47-3.68)
	C 4b Glu	4(2)	4(2)	NS	1.00 (0.24-4.12)	0(0)	3(2)	NS	0.14 (0.01-2.72)
	C 4a Glu	15(7)	10(5)	NS	1.59 (0.68-3.73)	12(7)	17(10)	NS	0.67 (0.30-1.48)
	C 4b Asp	10(5)	23(11)	NS	0.37 (0.17-0.83)	14(8)	24(14)	NS	0.52 (0.25-1.07)
	X^2-test	10.03		NS		9.57		NS	

Abbreviations: PR: poor responders; GR: good responders

The frequencies are shown as % (*n*). OD: *Odds ratio.* CI: 95 % confidence interval

P were considered significant when<0.05 (genotypes/alleles) or<0.05/6=0.0083 (haplotypes)

Table 3 Genotype, allele and haplotype frequencies for *BDKRB2* gene in hypertensive patients classified as poor or good responders to enalapril 10 or 20 mg/day

		Enalapril 10 mg				Enalapril 20 mg			
		PR (n=24)	GR (n=24)	*P*-value	OD (CI)	PR (n=29)	GR (n=29)	*P*-value	OD (CI)
$C^{-58}T$	CC	33(8)	33(8)	-	1.00 (reference)	41 (12)	38 (11)	-	1.00 (reference)
	CT	46(11)	58(14)	NS	0.79 (0.43-1.47)	38 (11)	52 (15)	NS	0.68 (0.37-1.24)
	TT	21(5)	8(2)	0.042	2.63 (1.02-6.77)	21 (6)	10 (3)	NS	1.95 (0.81-1.67)
	X^2-test	7.21		0.027		6.20		0.045	
	C	56(27)	63(30)	-	1.00 (reference)	60 (35)	64 (37)	-	1.00 (reference)
	T	44(21)	38(18)	NS	1.28 (0.73-2.26)	40 (23)	36 (21)	NS	1.19 (0.67-2.10)
Exon 1 +9/-9	+9/+9	29(7)	25(6)	-	1.00 (reference)	28 (8)	28 (8)	-	1.00 (reference)
	+9/-9	38(9)	54(13)	NS	0.61 (0.31-1.19)	52 (15)	59 (17)	NS	0.88 (0.46-1.68)
	-9/-9	33(8)	21(5)	NS	1.36 (0.63-2.91)	21 (6)	14 (4)	M	1.50 (0.64-3.53)
	X^2-test	5.75		NS		1.841		NS	
	+9	48(23)	52(25)	-	1.00 (reference)	53 (31)	57 (33)	-	1.00 (reference)
	- 9	52(25)	48(23)	NS	1.17 (0.67-2.04)	47 (27)	43 (25)	NS	1.18 (0.67-2.05)
Haplotypes									
	C -9	31(15)	35(17)	NS	0.83 (0.46-1.51)	34 (20)	28 (16)	NS	1.33 (0.73-2.42)
	T -9	21(10)	13(6)	NS	1.78 (0.84-3.79)	12 (7)	16 (9)	NS	0.72 (0.32-1.60)
	C +9	25(12)	27(13)	NS	0.90 (0.48-1.70)	26 (15)	36 (21)	NS	0.62 (0.34-1.14)
	T +9	23(11)	25(12)	NS	0.90 (0.47-1.72)	28 (16)	21 (12)	NS	1.46 (0.76-2.80)
	X^2-test	2.29		NS		3.76		NS	

Abbreviations: PR: poor responders; GR: good responders

The frequencies are shown as % (*n*). OD: *Odds ratio.* CI: 95 % confidence interval

P were considered significant when<0.05 (genotypes/alleles) or<0.05/4=0.0125 (haplotypes)

associated with modulation of pharmacologic treatment in patients with preeclampsia[48] and erectile dysfunction [32], we did not observe associations of haplotypes with the response to enalapril.

In addition to the eNOS gene, the *BDKRB2* gene is other good candidate as predictor of the ACE inhibitor response, because the activation of the bradykinin receptor B2 is related to NO formation pathway [49]. In this regard, our results suggest that TT carriers of the *BDKRB2* C^{-58} T polymorphism had worse responses to antihypertensive therapy.

On the other hand, the T allele has been associated with increased *BDKRB2* gene transcription in vitro [26], which is consistent with a protective effect against hypertension exhibited by this genetic variant in Asians and African–Americans [30, 50]. Although the presence of this allele

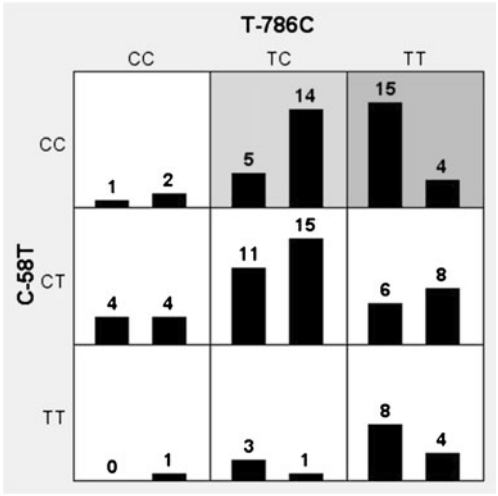

Fig. 3 Robust multifactor dimensionality reduction (RMDR) best model for the interaction between the *eNOS* $T^{-786}C$ and *BDKRB2* $C^{-58}T$ polymorphisms in hypertensive patients classified according to response to enalapril. The distributions of poor responders (left bars) and good responders (right bars) are illustrated for each combination of genotypes. The light grey cells are labeled as better response to enalapril, dark grey cells are labeled as worse response to enalapril, and white cells are labeled as unknown

also has been associated with the appearance of ACE inhibitor-related cough in Japanese patients [50, 51], other studies involving different populations did not show any association [52–54].

Our findings showed that TT carriers for the $C^{-58}T$ polymorphism also had lower baseline systolic and diastolic blood pressure. Altogether, our results suggest that although carriers of C allele may have higher blood pressure levels, they seem to have increased benefits from the treatment with enalapril. We have not found any other association between antihypertensive response to enalapril and BE1 +9/-9 polymorphism or *BDKRB2* haplotypes. These findings are in line with previous results showing that although *eNOS* haplotypes may be associated with susceptibility to hypertension [24], they are not associated with resistance to antihypertensive therapy [55].

Interactions between *eNOS* and *BDKRB2* genetic variants were also expected, as both proteins are involved in the same physiological pathway described above. While neither the CC genotype nor the C allele for the $C^{-58}T$ *BDKRB2* polymorphism were significantly associated with the response to enalapril at single–locus analysis, gene–gene interaction analysis showed that the $C^{-58}T$ CC genotype combined with *eNOS* $T^{-786}C$ TT genotype was more frequent in poor responders, while the combination of $C^{-58}T$ CC genotype with *eNOS* TC genotype was more frequent among good responders (Fig. 3). These findings are obscured when single BDKRB2 genotypes alone are considered, thus highlighting the importance of gene–gene interactions to the genetic component of drug responses (39, 40).

Enalapril had no effects on the circulating concentrations of markers of endogenous NO formation in a previous study [56], although *eNOS* polymorphisms apparently contribute to the responses to enalapril. This is probably explained by the fact that enalapril promotes increased NO formation at tissue levels that are not clearly reflected by the circulating levels markers of NO formation, as previously suggested [56].

As limitations of our study, we should mention the low number of patients included and that we were not able to stratify analysis by race, due to the low number of black subjects. However, given the sample size, we were able to obtain significant results with 80 % statistical power, therefore this study is not underpowered. Ethnicity may be a concern, as there are notable differences in *eNOS* and *BDKRB2* genotypes distributions between white and black subjects [37, 57]. However, there were no inter-ethnic differences between poor and good responders to enalapril in this study. Other issues that have not been addressed in the present study include bradykinin receptor polymorphisms that may predispose to side effects resulting from the use of ACE inhibitors, differences between ACE inhibitors, and other genetic polymorphisms that could affect the responses to these drugs.

In conclusion, our findings suggest that both *eNOS* and *BDKRB2* genotypes may affect the antihypertensive responses to enalapril. The TC and CC genotypes (*eNOS* $T^{-786}C$ polymorphism) are associated with better antihypertensive responses to enalapril, while the TT genotype of *BDKRB2* gene is associated with worse responses to this ACE inhibitor. Both genes may interact and affect enalapril responses, as CC genotype in *BDKRB2* is associated with good or poor responses depending on the *eNOS* $T^{-786}C$ genotypes.

Table 4 Robust multifactor dimensionality reduction (RMDR) multilocus models when compared poor responders (PR) and good responders (GR) to enalapril

Interaction Models	Training Score	Testing Score	CVC[a]	P
$T^{-786}C$ *eNOS*	0.6229	0.5099	8/10	0.7705
$T^{-786}C$ *eNOS*; $C^{-58}T$ *BDKRB2*	0.6580	0.6580	10/10	0.0450*
Intron4 *eNOS*; $C^{-58}T$ and BE1+9-9 *BDKRB2*	0.6933	0.5394	9/10	0.6060
$T^{-786}C$ and Intron4 *eNOS*; $C^{-58}T$ and BE1+9-9 *BDKRB2*	0.6429	0.4583	6/10	0.9550

[a] CVC: Cross Validation Consistency; * $P<0.05$ statistically significant, after 1.000 permutations

Acknowledgments This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP).

References

1. Lawes CM, Vander Hoorn S, Rodgers A (2008) Global burden of blood-pressure-related disease, 2001. Lancet 371(9623):1513–1518
2. Linz W, Wohlfart P, Scholkens BA, Malinski T, Wiemer G (1999) Interactions among ACE, kinins and NO. Cardiovasc Res 43 (3):549–561
3. Mason RP, Cockcroft JR (2006) Targeting nitric oxide with drug therapy. J Clin Hypertens (Greenwich) 8(12 Suppl 4):40–52
4. Moreau ME, Garbacki N, Molinaro G, Brown NJ, Marceau F, Adam A (2005) The kallikrein-kinin system: current and future pharmacological targets. J Pharmacol Sci 99(1):6–38
5. Bachetti T, Comini L, Pasini E, Cargnoni A, Curello S, Ferrari R (2001) Ace-inhibition with quinapril modulates the nitric oxide pathway in normotensive rats. J Mol Cell Cardiol 33(3):395–403
6. Comini L, Bachetti T, Cargnoni A, Bastianon D, Gitti GL, Ceconi C, Ferrari R (2007) Therapeutic modulation of the nitric oxide: all ace inhibitors are not equivalent. Pharmacol Res 56(1):42–48
7. De Gennaro CV, Rigamonti A, Fioretti S, Bonomo S, Manfredi B, Ferrario P, Bianchi M, Berti F, Muller EE, Rossoni G (2005) Angiotensin-converting enzyme inhibition and angiotensin AT1-receptor antagonism equally improve endothelial vasodilator function in L-NAME-induced hypertensive rats. Eur J Pharmacol 516 (3):253–259
8. Miguel-Carrasco JL, Monserrat MT, Mate A, Vazquez CM (2010) Comparative effects of captopril and l-carnitine on blood pressure and antioxidant enzyme gene expression in the heart of spontaneously hypertensive rats. Eur J Pharmacol 632(1–3):65–72
9. Hornig B, Kohler C, Drexler H (1997) Role of bradykinin in mediating vascular effects of angiotensin-converting enzyme inhibitors in humans. Circulation 95(5):1115–1118
10. Joshi MS, Mineo C, Shaul PW, Bauer JA (2007) Biochemical consequences of the NOS3 Glu298Asp variation in human endothelium: altered caveolar localization and impaired response to shear. FASEB J 21(11):2655–2663
11. Miyamoto Y, Saito Y, Nakayama M, Shimasaki Y, Yoshimura T, Yoshimura M, Harada M, Kajiyama N, Kishimoto I, Kuwahara K, Hino J, Ogawa E, Hamanaka I, Kamitani S, Takahashi N, Kawakami R, Kangawa K, Yasue H, Nakao K (2000) Replication protein A1 reduces transcription of the endothelial nitric oxide synthase gene containing a -786T–> C mutation associated with coronary spastic angina. Hum Mol Genet 9(18):2629–2637
12. Nakayama M, Yasue H, Yoshimura M, Shimasaki Y, Kugiyama K, Ogawa H, Motoyama T, Saito Y, Ogawa Y, Miyamoto Y, Nakao K (1999) T-786–> C mutation in the 5'-flanking region of the endothelial nitric oxide synthase gene is associated with coronary spasm. Circulation 99(22):2864–2870
13. Zhang MX, Zhang C, Shen YH, Wang J, Li XN, Chen L, Zhang Y, Coselli JS, Wang XL (2008) Effect of 27nt small RNA on endothelial nitric-oxide synthase expression. Mol Biol Cell 19(9):3997–4005
14. Zhang MX, Zhang C, Shen YH, Wang J, Li XN, Zhang Y, Coselli J, Wang XL (2008) Biogenesis of short intronic repeat 27-nucleotide small RNA from endothelial nitric-oxide synthase gene. J Biol Chem 283(21):14685–14693
15. Metzger IF, Ishizawa MH, Rios-Santos F, Carvalho WA, Tanus-Santos JE (2011) Endothelial nitric oxide synthase gene haplotypes affect nitrite levels in black subjects. Pharmacogenomics J 11(6):393–399
16. Metzger IF, Sertorio JT, Tanus-Santos JE (2007) Modulation of nitric oxide formation by endothelial nitric oxide synthase gene haplotypes. Free Radic Biol Med 43(6):987–992
17. Metzger IF, Souza-Costa DC, Marroni AS, Nagassaki S, Desta Z, Flockhart DA, Tanus-Santos JE (2005) Endothelial nitric oxide synthase gene haplotypes associated with circulating concentrations of nitric oxide products in healthy men. Pharmacogenet Genomics 15(8):565–570
18. Hyndman ME, Parsons HG, Verma S, Bridge PJ, Edworthy S, Jones C, Lonn E, Charbonneau F, Anderson TJ (2002) The T-786–> C mutation in endothelial nitric oxide synthase is associated with hypertension. Hypertension 39(4):919–922
19. Nejatizadeh A, Kumar R, Stobdan T, Goyal AK, Sikdar S, Gupta M, Javed S, Pasha MA (2008) Endothelial nitric oxide synthase gene haplotypes and circulating nitric oxide levels significantly associate with risk of essential hypertension. Free Radic Biol Med 44(11):1912–1918
20. Sandrim VC, Palei AC, Cavalli RC, Araujo FM, Ramos ES, Duarte G, Tanus-Santos JE (2008) eNOS haplotypes associated with gestational hypertension or preeclampsia. Pharmacogenomics 9(10):1467–1473
21. Vasconcellos V, Lacchini R, Jacob-Ferreira AL, Sales ML, Ferreira-Sae MC, Schreiber R, Nadruz W, Tanus-Santos JE (2010) Endothelial nitric oxide synthase haplotypes associated with hypertension do not predispose to cardiac hypertrophy. DNA Cell Biol 29(4):171–176
22. Lee YC, Wu WJ, Liu CC, Wang CJ, Li WM, Huang CH, Yeh HC, Ke HL, Huang SP (2009) The associations among eNOS G894T gene polymorphism, erectile dysfunction, and benign prostate hyperplasia-related lower urinary tract symptoms. J Sex Med 6 (11):3158–3165
23. Napoli C, Ignarro LJ (2007) Polymorphisms in endothelial nitric oxide synthase and carotid artery atherosclerosis. J Clin Pathol 60 (4):341–344
24. Sandrim VC, Coelho EB, Nobre F, Arado GM, Lanchote VL, Tanus-Santos JE (2006) Susceptible and protective eNOS haplotypes in hypertensive black and white subjects. Atherosclerosis 186(2):428–432
25. Sandrim VC, de Syllos RW, Lisboa HR, Tres GS, Tanus-Santos JE (2006) Endothelial nitric oxide synthase haplotypes affect the susceptibility to hypertension in patients with type 2 diabetes mellitus. Atherosclerosis 189(1):241–246
26. Braun A, Kammerer S, Maier E, Bohme E, Roscher AA (1996) Polymorphisms in the gene for the human B2-bradykinin receptor. New tools in assessing a genetic risk for bradykinin-associated diseases. Immunopharmacology 33(1–3):32–35
27. Braun A, Maier E, Kammerer S, Muller B, Roscher AA (1996) A novel sequence polymorphism in the promoter region of the human B2-bradykinin receptor gene. Hum Genet 97(5):688–689
28. Dhamrait SS, Payne JR, Li P, Jones A, Toor IS, Cooper JA, Hawe E, Palmen JM, Wootton PT, Miller GJ, Humphries SE, Montgomery HE (2003) Variation in bradykinin receptor genes increases the cardiovascular risk associated with hypertension. Eur Heart J 24(18):1672–1680
29. Hallberg P, Lind L, Michaelsson K, Karlsson J, Kurland L, Kahan T, Malmqvist K, Ohman KP, Nystrom F, Melhus H (2003) B2 bradykinin receptor (B2BKR) polymorphism and change in left ventricular mass in response to antihypertensive treatment: results from the Swedish Irbesartan Left Ventricular Hypertrophy Investigation versus Atenolol (SILVHIA) trial. J Hypertens 21(3):621–624
30. Niu W, Qi Y, Gao P, Zhu D (2010) A meta-analysis of the bradykinin B2 receptor gene –58C/T polymorphism with hypertension. Clin Chim Acta 411(5–6):324–328
31. Lacchini R, Silva PS, Tanus-Santos JE (2010) A pharmacogenetics-based approach to reduce cardiovascular mortality with the prophylactic use of statins. Basic Clin Pharmacol Toxicol 106(5):357–361

32. Muniz JJ, Lacchini R, Rinaldi TO, Nobre YT, Cologna AJ, Martins AC, Tanus-Santos JE (2011) Endothelial nitric oxide synthase genotypes and haplotypes modify the responses to sildenafil in patients with erectile dysfunction. Pharmacogenomics J
33. Nagassaki S, Sertorio JT, Metzger IF, Bem AF, Rocha JB, Tanus-Santos JE (2006) eNOS gene T-786C polymorphism modulates atorvastatin-induced increase in blood nitrite. Free Radic Biol Med 41(7):1044–1049
34. Peskircioglu L, Atac FB, Erdem SR, Deveci S, Verdi H, Ozkardes H (2007) The association between intron 4 VNTR, E298A and IVF 23 + 10G/T polymorphisms of ecNOS gene and sildenafil responsiveness in patients with erectile dysfunction. Int J Impot Res 19(2):149–153
35. Van Guilder GP, Pretorius M, Luther JM, Byrd JB, Hill K, Gainer JV, Brown NJ (2008) Bradykinin type 2 receptor BE1 genotype influences bradykinin-dependent vasodilation during angiotensin-converting enzyme inhibition. Hypertension 51(2):454–459
36. Tanus-Santos JE, Desai M, Flockhart DA (2001) Effects of ethnicity on the distribution of clinically relevant endothelial nitric oxide variants. Pharmacogenetics 11(8):719–725
37. Pretorius MM, Gainer JV, Van Guilder GP, Coelho EB, Luther JM, Fong P, Rosenbaum DD, Malave HA, Yu C, Ritchie MD, Vaughan DE, Brown NJ (2008) The bradykinin type 2 receptor BE1 polymorphism and ethnicity influence systolic blood pressure and vascular resistance. Clin Pharmacol Ther 83(1):122–129
38. Menashe I, Rosenberg PS, Chen BE (2008) PGA: power calculator for case-control genetic association analyses. BMC Genet 9:36
39. Pander J, Wessels JA, Mathijssen RH, Gelderblom H, Guchelaar HJ (2011) Pharmacogenetics of tomorrow: the 1 + 1 = 3 principle. Pharmacogenomics 11(7):1011–1017
40. Ritchie MD, Motsinger AA (2005) Multifactor dimensionality reduction for detecting gene-gene and gene-environment interactions in pharmacogenomics studies. Pharmacogenomics 6(8):823–834
41. Gui J, Andrew AS, Andrews P, Nelson HM, Kelsey KT, Karagas MR, Moore JH (2011) A robust multifactor dimensionality reduction method for detecting gene-gene interactions with application to the genetic analysis of bladder cancer susceptibility. Ann Hum Genet 75(1):20–28
42. Abe K, Nakayama M, Yoshimura M, Nakamura S, Ito T, Yamamuro M, Sakamoto T, Miyamoto Y, Yoshimasa Y, Saito Y, Nakao K, Yasue H, Ogawa H (2005) Increase in the transcriptional activity of the endothelial nitric oxide synthase gene with fluvastatin: a relation with the -786T > C polymorphism. Pharmacogenet Genomics 15(5):329–336
43. Mason RP, Jacob RF, Kubant R, Jacoby A, Louka F, Corbalan JJ, Malinski T (2012) Effects of angiotensin receptor blockers on endothelial nitric oxide release - The role of eNOS variants. Br J Clin Pharmacol
44. Souza-Costa DC, Sandrim VC, Lopes LF, Gerlach RF, Rego EM, Tanus-Santos JE (2007) Anti-inflammatory effects of atorvastatin: modulation by the T-786C polymorphism in the endothelial nitric oxide synthase gene. Atherosclerosis 193(2):438–444
45. Nagassaki S, Herculano RD, Graeff CF, Tanus-Santos JE (2009) eNOS T-786C polymorphism affects atorvastatin-induced changes in erythrocyte membrane fluidity. Eur J Clin Pharmacol 65(4):385–392
46. Tanus-Santos JE, Desai M, Deak LR, Pezzullo JC, Abernethy DR, Flockhart DA, Freedman JE (2002) Effects of endothelial nitric oxide synthase gene polymorphisms on platelet function, nitric oxide release, and interactions with estradiol. Pharmacogenetics 12(5):407–413
47. Zago AS, Kokubun E, Fenty-Stewart N, Park JY, Attipoe S, Hagberg J, Brown M (2010) Effect of physical activity and t-786C polymorphism in blood pressure and blood flow in the elderly. Arq Bras Cardiol 95(4):510–516
48. Sandrim VC, Palei AC, Luizon MR, Izidoro-Toledo TC, Cavalli RC, Tanus-Santos JE (2010) eNOS haplotypes affect the responsiveness to antihypertensive therapy in preeclampsia but not in gestational hypertension. Pharmacogenomics J 10(1):40–45
49. Erdos EG, Tan F, Skidgel RA (2010) Angiotensin I-converting enzyme inhibitors are allosteric enhancers of kinin B1 and B2 receptor function. Hypertension 55(2):214–220
50. Mukae S, Aoki S, Itoh S, Iwata T, Ueda H, Katagiri T (2000) Bradykinin B(2) receptor gene polymorphism is associated with angiotensin-converting enzyme inhibitor-related cough. Hypertension 36(1):127–131
51. Mukae S, Itoh S, Aoki S, Iwata T, Nishio K, Sato R, Katagiri T (2002) Association of polymorphisms of the renin-angiotensin system and bradykinin B2 receptor with ACE-inhibitor-related cough. J Hum Hypertens 16(12):857–863
52. Lee YJ, Tsai JC (2001) Angiotensin-converting enzyme gene insertion/deletion, not bradykinin B2 receptor -58T/C gene polymorphism, associated with angiotensin-converting enzyme inhibitor-related cough in Chinese female patients with non-insulin-dependent diabetes mellitus. Metabolism 50(11):1346–1350
53. Woo SW, Bang S, Chung MW, Jin SK, Kim YS, Lee SH (2009) Lack of association between ACE and bradykinin B2 receptor gene polymorphisms and ACE inhibitor-induced coughing in hypertensive Koreans. J Clin Pharm Ther 34(5):561–567
54. Grilo A, Saez-Rosas MP, Santos-Morano J, Sanchez E, Moreno-Rey C, Real LM, Ramirez-Lorca R, Saez ME (2011) Identification of genetic factors associated with susceptibility to angiotensin-converting enzyme inhibitors-induced cough. Pharmacogenet Genomics 21(1):10–17
55. Sandrim VC, Yugar-Toledo JC, Desta Z, Flockhart DA, Moreno H Jr, Tanus-Santos JE (2006) Endothelial nitric oxide synthase haplotypes are related to blood pressure elevation, but not to resistance to antihypertensive drug therapy. J Hypertens 24(12):2393–2397
56. Silva PS, Fontana V, Palei AC, Sertorio JT, Biagi C, Tanus-Santos JE (2011) Antihypertensive effects exerted by enalapril in mild to moderate hypertension are not associated with changes in the circulating levels of nitric oxide-related markers. Eur J Clin Pharmacol 67(4):365–370
57. Marroni AS, Metzger IF, Souza-Costa DC, Nagassaki S, Sandrim VC, Correa RX, Rios-Santos F, Tanus-Santos JE (2005) Consistent interethnic differences in the distribution of clinically relevant endothelial nitric oxide synthase genetic polymorphisms. Nitric Oxide 12(3):177–182