Genetic variants of the renin-angiotensin system and cardiovascular disease in peritoneal dialysis patients *

Andrzej KSIĄŻEK*
Wojciech ZAŁUSKA
Monika BURACZYŃSKA

Department of Nephrology, Medical University of Lublin, Lublin, Poland
Chair:
Prof. *Andrzej Książek*, M.D., Ph.D., Dr h.c. mult.

Mortality rate in end-stage renal disease (ESRD) patients is very high, due mainly to cardiovascular disease (CVD). Genetic variants of the renin-angiotensin system genes, angiotensin I – converting enzyme (ACE), angiotensinogen (AGT) and angiotensin II type 1 receptor (AT1R) are established candidates in CVD . The purpose of our study was to evaluate role of these polymorphisms in CVD in peritoneal dialysis (PD) patients. We examined 80 PD patients diagnosed with CVD and 80 PD patients without any symptoms or signs of CVD. Patients were genotyped for the ACE, AGT and AT1R polymorphisms by polymerase chain reaction (PCR), followed by restriction enzyme analysis. The genotype and allele frequencies were similar in PD patients with CVD and those without CVD. We analyzed combinations of risk alleles of studied polymorphisms. Genotypes with risk alleles of both ACE and AT1R polymorphisms were found in 27 % of patients with CVD, and with the risk alleles of both AT1R and AGT in 24 % of CVD patients. Both ACE D allele and AGT T allele were observed in 72 % of CVD patients. This combination of alleles was present in 46 % of patients without CVD (p < 0.0001). This is the first study to evaluate the impact of the renin-angiotensin system gene polymorphisms on CVD in PD patients. The simultaneous presence of D allele of ACE I/D polymorphism and T allele of AGT M235T polymorphism might be a strong predictor of a higher risk of developing CVD in dialyzed patients.

(NEPROL. DIAL. POL. 2015, 19, 112-115)

Słowa kluczowe:
• choroby układu sercowo-naczyniowego
• genotyp
• dializa otrzewnowa
• geny układu renina-angiotensyna
• allel ryzyka

Key words:
• cardiovascular disease
• genotyping
• peritoneal dialysis
• renin-angiotensin system genes
• risk alleles

Warianty genów układu renina-angiotensyna i choroby układu sercowo-naczyniowego u pacjentów leczonych dializą otrzewnową

Śmiertelność wśród pacjentów ze schyłkową niewydolnością nerek (ESRD) jest bardzo wysoka, co spowodowane jest głównie chorobami układu sercowo-naczyniowego (CVD). Warianty genetyczne genów układu renina-angiotensyna, enzymu konwertazy angiotensyny I (ACE), angiotensynogenu (AGT) i receptora typu 1 angiotensyny II (AT1R), są uznanymi kandydatami w chorobach układu sercowo-naczynioego. Celem niniejszego badania była ocena roli wymienionych polimorfizmów w CVD u pacjentów dializowanych otrzewnowo. Badano 80 pacjentów dializowanych otrzewnowo, ze zdiagnozowaną chorobą układu krążenia i 80 pacjentów bez objawów chorób układu krążenia. W DNA pacjentów oznaczano genotypy polimorfizmów genów ACE, AGT i AT1R metodą reakcji łańcuchowej polimerazy (PCR) i analizy z endonukleazami restrykcyjnymi. Częstości genotypów i alleli były podobne u pacjentów z CVD i pacjentów bez CVD. Analizowano kombinacje alleli ryzyka badanych polimorfizmów. Genotypy z allelami ryzyka dwóch polimorfizmów, ACE i AT1R, obserwowano u 27 % pacjentów z CVD, a z allelami ryzyka AT1R i AGT u 24 % pacjentów z CVD. Oba allele, ACE D i AGT T, obserwowano u 72 % pacjentów z chorobami układu sercowo-naczyniowego. Ta kombinacja alleli ryzyka była obecna u 46 % pacjentów bez CVD (p < 0.0001). Jest to pierwsze badanie oceniające związek polimorfizmów układu renina-angiotensyna z chorobami układu sercowo-naczyniowego u pacjentów dializowanych otrzewnowo. Równoczesna obecność allelu D polimorfizmu I/D genu ACE i allelu T polimorfizmu M235T genu AGT może być silnym predyktorem zwiększonego ryzyka wystąpienia CVD u pacjentów leczonych dializą otrzewnową.

(NEFROL. DIAL. POL. 2015, 19, 112-115)

**Praca dedykowana Panu Profesorowi Bolesławowi Rutkowskiemu*

Introduction

Chronic kidney disease (CKD) is a major public health problem due to high prevalence (about 10 % of general population), reduction in life expectancy and quality of life of patients and enormous cost [1]. Despite rapid improvements in dialysis technology, the mortality rate in end-stage renal disease (ESRD) patients treated with dialysis is very high. The main cause of this increase is cardiovascular disease (CVD) [2,3]. Although traditional risk factors such as hypertension, diabetes, dyslipidemia, age and smoking are common in dialysis patients, they can only in part explain the high prevalence of CVD in these patients [4,5]. Patients on peritoneal dialysis (PD) are exposed to a high glucose intake and its metabolic side effects such as obesity, diabetes or hyperlipidemia, which predispose to ischemic heart disease. Also,

Adres do korespondencji:
Andrzej Książek
Department of Nephrology
Medical University of Lublin
Dr K. Jaczewskiego 8, 20-954 Lublin, Poland
tel: +48 (81) 7244 538
fax: +48 (81) 7244 357
e-mail: ksan@interia.pl

an oxidative stress contributes to cardiovascular diseases in these patients [6,7]. However, the pathogenesis of cardiovascular disease in peritoneal dialysis patients is not completely understood.

As in other multifactorial diseases, genetic factors interact with environmental factors in a development of chronic renal failure and its complications. With the advent of genome-wide association scans, numerous risk variants have been identified as candidates for conferring susceptibility to renal and cardiovascular diseases but most of them with only modest effects [8,9].

Genetic variants of the renin-angiotensin system genes, encoding angiotensin I – converting enzyme (ACE), angiotensinogen (AGT) and angiotensin II type 1 receptor (AT1R) are established candidates for genetic factors in cardiovascular disease [10].

The purpose of our study was to evaluate a role of the ACE, AGT and AT1R gene polymorphisms in CVD in peritoneal dialysis patients.

Patients and Methods

In this retrospective study, the study group included 80 adult patients from a single dialysis center, treated with peritoneal dialysis and diagnosed with cardiovascular disorders and 80 age-matched peritoneal dialysis patients without any symptoms or signs of cardiovascular disease. These patients were dialyzed with either continuous ambulatory PD (n = 100) or automated PD (n = 60). Cardiovascular disease was diagnosed and documented as one or the combination of several pathological states : congestive heart failure, left ventricular hypertrophy, angina pectoris, ischemic heart disease, myocardial infarction, peripheral arterial disease, ischemic cerebral stroke or atheromatous lesions, all confirmed by appropriate biochemical, radiographic, echocardiographic and vascular diagnostic criteria. There was a substantial overlap between categories. At the beginning of the study 62 patients in the CVD+ group and 55 patients in the CVD- group were hypertensive and receiving antihypertensive medications. Hypertension was defined as a systolic blood pressure > 140 mm Hg and diastolic blood pressure > 90 mm Hg and / or use of antihypertensive medication. The study protocol for genetic analysis was approved by an institutional ethics committee. The investigation conforms to the principles of the Declaration of Helsinki.

Determination of genotypes

Genomic DNA was isolated from all subjects from peripheral blood leukocytes by the standard method .

Angiotensin I-converting enzyme insertion/deletion genotype was determined using standard protocol [11]. Because of the preferential amplification of the D allele, each DD genotype was confirmed by using insertion-specific primers [12]. Amplification products had the size of 490 bp for the insertion allele and 190 bp for the deletion allele. For insertion-specific primers the product of 335 bp was obtained.

The angiotensinogen gene M235T polymorphism was detected by restriction typing of PCR product. The following primers were used: forward: 5'- CCGTTTGTGCAGGGCCTGGCTCTCT - 3' and reverse: 5' - CAGGGTGCTGTCCACACTGGACCCC - 3'. Genomic DNA (300 ng) was amplified in a final volume of 50 µl, containing 10 mM TRIS pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each dNTP, 1 µM of each primer and 2 U Taq polymerase (all reagents from MBI Fermentas, St. Leon-Rot, Germany). The initial denaturation at 95^{0}C was followed by 35 cycles of denaturation at 94^{0}C, annealing at 65^{0}C and elongation at 72^{0}C, 1 min each. Final extension was at 72^{0}C for 7 min. The PCR product was digested with Psy I restriction enzyme (MBI Fermentas, St.Leon-Rot, Germany) and DNA fragments were separated by electrophoresis in 2 % agarose gel. The M allele was detected as a band of 165 bp, whereas the mutated T allele showed two fragments, 141 bp and 24 bp.

The A1166C variant of the angiotensin II type 1 receptor gene was identified with primers: forward: 5' -GCAGCACTTCACTACCAAATGGGC - 3' and reverse: 5' - CAGGACAAAAGCAGGCTAGGGAGA - 3'. The reaction conditions were the same as for the AGT polymorphism, except for the annealing step which was at 55^{0}C. The PCR product was digested with Bsu RI restriction enzyme (MBI Fermentas, St. Leon-Rot, Germany), giving a 231 bp fragment for the C allele and undigested 255 bp fragment for the A allele.

Statistical analysis

Statistical calculations were performed using SPSS 11.0 for Windows (SPSS, Inc., Chicago, IL, USA). For baseline characteristics the normally distributed continuous variables are presented as means ± SD. The Hardy-Weinberg equilibrium was verified with the X^2 test. Genotype distribution and allele frequencies were compared between groups using a chi-squared test of and *z* statistics. For continuous variables the t-test and ANOVA were used for statistical significance. Where appropriate, the odds ratios (OR) with corresponding 95 % confidence intervals (CI) were calculated for the effects of high-risk alleles and genotypes. A two-tailed type I error rate of 5 % was considered statistically significant. Power calculations were done using on-line available power calculator (http://calculators.stat.ucla.edu).

Results

The demographic and laboratory data of studied subjects are presented in Table I. In the CVD+ group higher frequencies of hypertension and diabetes were observed. This group of patients also showed a tendency to higher LDL cholesterol and lower HDL cholesterol levels than in CVD- patients but the differences were not statistically significant. The hemoglobin level was higher in the CVD- group (p = 0.02).

The genotype distribution for all three polymorphisms was in agreement with Hardy-Weinberg equilibrium (chi-squared test, p = 0.131-0.995). The genotype and allele frequencies of studied polymorphisms were similar in peritoneal dialysis patients with CVD and those without it (Tab. II). The D allele of the ACE gene and the C allele of the AT1R gene polymorphisms were more frequent in patients with CVD (0.57 and 0.36 versus 0.49 and 0.29, respectively) but the differences were not statistically significant.

We next analyzed combinations of risk alleles of studied polymorphisms. The results are shown in Table III. Genotypes with the D allele of the ACE gene polymorphism and the C allele of AT1R polymorphism were found in 27 % of patients with CVD, and the AT1R C allele with AGT T allele in 24 % of CVD patients, The ACE D allele in combination with the AGT T allele was observed in 72 % of cardiovascular disease patients (p < 0.01). In the group without CVD this combination of the ACE and AGT risk alleles was present in 46 % of patients. No interaction was observed between ACE and AT1R or AGT and AT1R polymorphisms in increasing a risk of cardiovascular disease in peritoneal dialysis patients.

Discussion

Inflammation, malnutrition, calcification,

Table I
Clinical characteristics of studied subjects.
Charakterystyka kliniczna badanych osób.

	Peritoneal dialysis patients	
	CVD+ (n = 80)	CVD- (n = 80)
Age at study (yrs)	61.3 ± 16	60.5 ± 19
Male / female	49 / 31	43 / 37
CAPD (%)	52 (65)	48 (60)
Automated PD (%)	28 (35)	32 (40)
Time on dialysis (yrs)	3.1 ± 2.3	2.4 ± 1.7
Body mass index (kg/m^2)	27.2 ± 3.7	26.1 ± 3.4
Hypertension (%)	62 (77.5)	55 (68.7)
Diabetes (%)	14 (18)	10 (12.5)
Serum cholesterol (mg/dl)	173 ± 38	166 ± 39
HDL cholesterol (mg / dl)	53.7 ± 19.2	55.1 ± 17.6
LDL cholesterol (mg / dl)	132.6 ± 38	122.5 ± 32.4
Serum creatinine (mg/dl)	8.4 ± 2.6	8.8 ± 3.1
Hemoglobin (g/dl)	12.3 ± 2.1*	13.7 ± 1.8

CVD - cardiovascular disease. Values ± SD; percentages in parentheses; *p < 0.05

Table II
Distribution of alleles and genotypes in PD patients.
Rozkład alleli i genotypów u pacjentów dializowanych otrzewnowo.

	Peritoneal dialysis patients			OR (95 % CI)
Polymorphism	**CVD+ (n = 80)**	**CVD- (n = 80)**	***P* value**	**for risk allele**
ACE				
II	17 (21)	25 (31)	0.10	1.35 (0.87-2.10)
ID	35 (44)	31 (39)		
DD	28 (35)	24 (30)		
Alleles I / D	0.43 / 0.57	0.51 / 0.49		
AGT				
MM	20 (25)	19 (24)	1.00	1.0 (0.64-1.55)
MT	38 (47)	40 (50)		
TT	22 (28)	21 (26)		
Alleles M / T	0.49 / 0.51	0.49 / 0.51		
AT1R				
AA	33 (41)	40 (50)	0.12	1.33 (0.83-2.12)
AC	37 (46)	33 (41)		
CC	10 (13)	7 (9)		
Alleles A / C	0.64 / 0.36	0.71 / 0.29		

CVD - cardiovascular disease. Brackets indicate percentage.

Table III
Effect of two-locus risk allele combination on CVD development.
Wpływ kombinacji alleli ryzyka w dwóch loci na występowanie CVD.

Risk allele		**Fraction with**		**OR**
combination	**n (%)**	**3rd allele (%)**	***P* value**	**(95 % CI)**
- / D + - / C	22 (27)	19 (86)	0.20	0.86 (0.27-1.06)
- / C + - / T	19 (24)	19 (100)	0.055	0.91 (0.43-1.35)
- / D + - / T	58 (72)	19 (33)	< 0.0001	3.06 (1.58-5.92)

CVD - cardiovascular disease [-, other] allele for either homo- or heterozygote. 3rd allele, risk allele of the polymorphism not included in presented pair.

endothelial dysfunction and oxidative stress are cardiovascular disease risk factors that contribute to mortality in peritoneal dialysis patients [13]. Several reports suggested a role of candidate genes in the pathogenesis of cardiovascular disease in dialyzed patients [14]. The importance of evaluating different candidate genes lies in the possibility to identify risk factors allowing early identification of patients with renal failure at risk of developing serious cardiovascular disorders [15].

In this paper we evaluated a role of the ACE, AGT and AT1R gene polymorphisms in CVD in a group of 160 peritoneal dialysis patients. Although , tested separately, none of genotypes of three studied polymorphisms appears to be directly involved in a development of CVD in peritoneal dialysis patients, the simultaneous presence of risk alleles of the ACE and AGT polymorphisms is associated with CVD in the studied group. The odds ratio for the association of these two polymorphisms was 3.06 (95 % CI 1.58-5.92), suggesting an increased risk of cardiovascular disease. This observation might indicate an important combined risk factor for cardiovascular disease and it should be a subject of further study.

Although it has been suggested that ACE gene plays a role in the occurrence of coronary heart disease in the general population, the reports on its role in cardiovascular diseases are still controversial [16,17]. In our study the risk allele (D allele) of the ACE gene polymorphism alone did not have a significant effect on the occurrence of cardiovascular disease in peritoneal dialysis patients.

There were some other studies evaluating the effect of gene variants on CVD and clinical outcomes in peritoneal dialysis patients. In a Chinese study it was observed that the CRP gene genotype was associated with a cardiovascular event-free survival benefit among peritoneal dialysis patients with cholesterol levels of 200 mg/dl or greater. This finding indicated to a complex interaction among cholesterol, CRP and cardiovascular disease in peritoneal dialysis patients [18]. The same group also studied the relationship between glutathione S-transferase M1 polymorphism and clinical outcome in peritoneal dialysis patients. They found that GST M1 genotype was associated with better survival in elderly PD patients [19].

Genetic bases of cardiovascular diseases are complex and include effects of gene-gene and gene environment interactions [20,21]. Three important genes of the RAS system, ACE, AGT and AT1R, belonging to the same metabolic pathway, may interact with each other. ACE and AGT polymorphisms affect the amount of their final product and may potentially contribute to a high risk of CVD that is not found for those polymorphisms alone. Our observations on the interaction between ACE and AGT gene polymorphisms are in agreement with several published reports of such interactions between the RAS system genes [22-24]. In a study of 110 myocardial infarction (MI) patients with ACE, AGT and AT1R gene polymorphisms, the genotypic frequencies for three studied genes alone were similar between MI patients and controls. In genotypic combinations involving unfavorable alleles, the risk (odds ratio) of developing MI was 2.92 for AGT vs. ACE polymorphisms [25]. In another study, an interaction was also found between homozygotes for T235 of the AGT polymorphism and ACE DD genotype in developing coronary heart disease and MI [26]. An interaction was found between ACE gene and AGT haplotypes in the risk of hypertension [27].

As most of the association studies, ours has some potential limitations. The sample size in this study is small . A survival and selection bias cannot be excluded in this retrospective case-control study. In addition, comorbidities in peritoneal dialysis patients might represent a confounding factor. The strengths of the study are that all patients are of the same ethnic origin. Furthermore, all subjects were examined in a standardized manner, with well defined diagnostic criteria and genotyping was performed blind with respect to case-control status. However, the occurrence of renal diseases and comorbidities such as cardiovascular disease depends on the interaction among the different risk alleles, environmental factors and the lifestyle. The influence of single polymorphisms is rather small and an interactive effect of several factors may lead to an underestimation or overestimation of a role of given polymorphism in determining the phenotype.

In conclusion, to our knowledge ours is the first study to evaluate the impact of the renin-angiotensin system gene polymorphisms on cardiovascular disease in peritoneal dialysis patients. The results of our study indicate that simultaneous presence of the D allele of the ACE gene I/D polymorphism and the T allele of the AGT gene M235T polymorphism might be a strong predictor of a higher risk of developing cardiovascular disease in dialyzed patients.

Disclosures

The authors do not have any conflicts of interest to declare.

Acknowledgments

This study was supported by grants DS 379/12 (MB) and DS 383/12 (AK) from Medical University of Lublin. The authors thank the Peritoneal Dialysis Team for referring the patients and supplying clinical data.

References

1. **El Nahas M, Bello AK**: Chronic kidney disease : the global challenge. Lancet 2005; 365: 331-340.
2. **Cheung AK, Sarnak MJ, Yan C**: Cardiac diseases in maintenance hemodialysis patients: results of the

HEMO study. Kidney Int. 2004; 65: 2380-2389.
3. **McCullough PA, Li S, Jurkovitz CT, Stevens L, Collins AJ, Chen SC et al**: Chronic kidney disease, prevalence of premature cardiovascular disease, and relationship to short-term mortality. Am Heart J. 2008; 156: 277-283.
4. **Locatelli F, Covic A, Chazot C, Leunissen K, Luno J, Yaqoob M**: Hypertension and cardiovascular risk assessment in dialysis patients. Nephrol Dial Transplant. 2004; 19: 1058-1068.
5. **Yao Q, Lindholm B, Stenvinkel P**: Inflammation as a cause of malnutrition, atherosclerotic cardiovascular disease, and poor outcome in hemodialysis patients. Hemodial Int. 2004; 8: 118-129.
6. **Ducloux D, Bresson-Vautrin C, Kribs M, Abdelfatah A, Chalopin JM**: C-reactive protein and cardiovascular disease in peritoneal dialysis patients. Kidney Int. 2002; 62: 1417-1422.
7. **Choi HY, Lee JE, Han SH, Yoo TH, Kim BS. et al**: Association of inflammation and protein-energy wasting with endothelial dysfunction in peritoneal dialysis patients. Nephrol Dial Transplant. 2010; 25: 1266-1271.
8. **Böger CA, Heid IM**: Chronic kidney disease : novel insights from genome - wide association studies. Kidney Blood Press Res. 2011; 34: 225-234.
9. **Roberts N, Chen L, Wells GA, Stewart AF**: Recent success in the discovery of coronary artery disease genes. Can J Physiol Pharmacol. 2011; 89: 609-615.
10. **Gluba A, Banach M, Mikhailidis DP, Rysz J**: Genetic determinants of cardiovascular disease: the renin-angiotensin-aldosterone system, paraoxonases, endothelin-1, nitric oxide synthase and adrenergic receptors. In Vivo 2009; 23: 797-812.
11. **Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F**: An insertion/deletion polymorphism in the angiotensin I - converting enzyme gene accounting for half the variance of serum enzyme levels. J Clin Invest. 1990; 86: 1343-1346.
12. **Lindpaintner K, Pfeffer MA, Kreutz R, Stampfer MJ, Grodstein F. et al**: A prospective evaluation of an angiotensin-converting enzyme gene polymorphism and the risk of ischemic heart disease. N Engl J Med. 1995; 322: 706-711.
13. **Balafa O, Krediet RT**: Peritoneal dialysis and cardiovascular disease. Minerva Urol Nephrol. 2012; 64: 153-162.
14. **Pernod G, Bosson JL, Goishayan D, Barro C, Fomeris G. et al**: Phenotypic and genotypic risk factors for cardiovascular events in an incident dialysis cohort. Kidney Int. 2006; 69: 1424-1430.
15. **Axelsson J, Devuyst O, Nordfors L, Heimbürger O, Stenvinkel P, Lindholm B**: Place of genotyping and phenotyping in understanding and potentially modifying outcomes in peritoneal dialysis patients. Kidney Int 2006; 103 (Suppl.): S138-145.
16. **Sayed-Tabatabaei FA, Oostra BA, Isaacs A, van Duijn CM, Witteman JCM**: ACE polymorphisms. Circulation Res. 2006; 98: 1123-1133.
17. **Bai Y, Wang L, Hu S, Wei Y**: Association of angiotensin-converting enzyme I/D polymorphism with heart failure : a meta-analysis. Mol Cell Biochem. 2012; 361: 297-304.
18. **Poon PY, Szeto CC, Kwan BC, Chow KM, Li PK**: Relationship between CRP polymorphism and cardiovascular events in Chinese peritoneal dialysis patients. Clin J Am Soc Nephrol. 2012; 7: 304-309.
19. **Poon PY, Szeto CC, Kwan BC, Chow KM, Li PK**: Relationship between glutathione S-transferase M1 polymorphism and clinical outcomes in Chinese peritoneal dialysis patients. J Nephrol. 2012; 25: 310-316.
20. **Ho E, Bhindi R, Ashley EA, Figtree GA**: Genetic analysis in cardiovascular disease. Cardiol Rev. 2011; 19: 81-89.
21. **Chan SY, White K, Loscalzo J**: Deciphering the molecular basis of human cardiovascular disease through network biology. Curr Opin Cardiol. 2012; 27: 202-209.
22. **Tsai CT, Hwang JJ, Chiang FT, Wang YC, Tseng CD. et al**: Renin - angiotensin system gene polymorphisms and atrial fibrillation: A regression approach for the detection of gene-gene interactions in a large hospitalized population. Cardiology 2008; 111: 1-7.
23. **Tsai CT, Hwang JJ, Wu CK, Lee JK, Tseng CD. et al**: Polygenic regression model of renin-angiotensin system genes and the risk of coronary artery disease in a large angiographic population. Clin Chim Acta 2011; 412: 619-624.
24. **Mehri S, Mahjoub S, Hammami S, Zaroui A, Frih A. et al**: Renin-angiotensin system polymorphisms in relation to hypertension status and obesity in a Tunisian population. Mol Biol Rep. 2012; 39: 4059-4065.
25. **Araujo MA, Goulart LR, Cordeiro ER, Gatti RR, Menezes BS. et al**: Genotypic interactions of renin-angiotensin system genes in myocardial infarction. Int J Cardiol. 2005; 103: 27-32.
26. **Ludwig EH, Borecki IB, Ellison RC, Folsom A, Heiss G. et al**: Associations between candidate loci angiotensin-converting enzyme and angiotensinogen with coronary heart disease and myocardial infarction: the NHLBI Family Heart Study. Ann Epidemiol. 1997; 7: 3-12.
27. **Tsai CT, Fallin D, Chiang FT, Hwang JJ, Lai LP. et al**: Angiotensinogen gene haplotype and hypertension interaction with ACE gene I allele. Hypertension 2003; 41: 9-15.