Renin Genetic Polymorphism in Heart Failure Patients

DANA POP^{1,2}, ADELA-VIVIANA SITAR-TĂUT^{1,3}, LUCIA PROCOPCIUC¹,
MIRELA CEBANU², M. ZDRENGHEA¹, D. ZDRENGHEA^{1,2}

¹“Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca, Romania

²Rehabilitation Hospital, Cardiology Department, Cluj-Napoca, Romania

³New Bluelife, Cluj-Napoca, Romania

Background. Genetic polymorphism of renin-angiotensin-aldosterone system affects the pathogenesis of hypertension (HTN), ischemic heart disease (IHD) and heart failure (HF). The purpose of our study is to analyze A/G renin genetic polymorphism in heart failure patients.

Methods. We investigated renin polymorphism in 83 subjects hospitalized in the Cardiology Department of the Rehabilitation Hospital Cluj-Napoca, using the PCR amplification method. 43 patients were diagnosed with heart failure [NYHA III-IV class], and 40 subjects without cardiovascular disease (control group). The NT-proBNP and the presence of cardiovascular risk factors were assessed.

Results. Heart failure etiology was IHD in 60.46 % of patients. The average value of NT-pro BNP was 2991.24 ± 2034.6 pg/ml. As it was expected, HF patients presented low lipid levels: total cholesterol = 162.36 ± 38.28 mg/dl, LDL-Cholesterol = 104.88 ± 27.60 mg/dl, triglycerides = 109.12 ± 55.84 mg/dl, HDL-Co = 35.68 ± 9.55 mg/dl. A/G renin genetic polymorphism [with pathogenic potential] in heart failure patients was of 60.46% (homozygote 4.65% and heterozygote 55.81%). Conversely, pathogenic mutations were found only in 38.46% of hypertensive patients, but also in 55.88% and 22.22% patients with obesity/overweight and diabetes. The heterozygote form was found in only 37.5% of control subjects.

Conclusion. This study showed no involvement of A/G renin polymorphisms in the pathogenesis of HF.

Key words: renin genetic polymorphism, heart failure.

INTRODUCTION

Genetic predisposition in cardiovascular pathology is the consequence of the interaction between genetic polymorphism and external risk factors. At the same time, the presence of the genetic mutations influence the therapeutic and diet response in patients diagnosed with cardiovascular disease. Genetic mutations of renin-angiotensin-aldosterone (RAS) system (renin, angiotensinogen, angiotensin II converting enzyme, angiotensin II receptors type 1 and 2) represent a great interest for many researchers because of their key role in pathogenesis of chronic heart disease. RAS system is activated during the last stages of chronic heart failure and leads to hydro saline retention with an increase of cardiac input. Discovered 100 years ago, renin is one of the most important hormones involved in the pathogenesis of high blood pressure, as well as of other cardiac diseases. Secreted by the juxtaglomerular renal cells, renin is an aspartyl protease which carries out the conversion of angiotensinogen released by the liver to angiotensin I. Renin is an aspartyl protease which carries out the

conversion of angiotensinogen released by the liver to angiotensin I. Angiotensin I is subsequently converted to angiotensin II by the angiotensin converting enzyme found in the lungs. Also, it was reported that non-ACE pathways are involved [1, 2]. Excessive renin secretion causes high angiotensin II and aldosterone plasma levels with vasoconstriction and hydro saline retention effects, both having a significant contribution in the progression of chronic heart failure [3].

OBJECTIVES

The purpose of our study is to analyze the presence of A/G renin genetic polymorphism in heart failure patients, a less discussed aspect in specialized studies.

MATERIAL AND METHOD

This study was performed on 83 subjects admitted in the Rehabilitation Hospital Cluj-

Napoca, Cardiology Department. 43 patients were diagnosed with chronic heart failure NYHA III-IV class, according to 2008 European Society of Cardiology criteria [4] and 40 subjects without cardiovascular disease [control group]. The mean age in patients with chronic heart failure was 64.96 ± 13.19 years and 69.76% of them were men. For all patients the cardiovascular risk factors were assessed, as well as NT-proBNP (normal value being exclusion criteria) in all patients and also they were assessed for the presence of cardiovascular risk factors. In adults, overweight was defined as a body mass index (BMI) from 25 to 29 kg/m^2 and obesity as a $\text{BMI} \geq 30 \text{ kg/m}^2$ [5]. Levels of NT-proBNP were measured using the BNP Fragment EIA kit (Biomedica; Vienna, Austria).

We examined polymorphisms the G/AI 9-83 transition in intron 8 of the renin gene (REN). The REN genes were amplified by polymerase chain reaction (PCR) using the following pairs of primers (method described by Rupert, which was modified in the biochemistry laboratory from "Iuliu Hațieganu" University of Medicine and Pharmacy, Cluj-Napoca) [6, 7]:

83A/G-REN Forward: 5'- GAGGTCGAGTCGGC
CCCCCT-3'

Reverse: 5'- TGCCCCAAACATGGCCA CACAT-3'

An amount of 25 ng genomic DNA was amplified in a total volume of 25 μl , containing 0.2 μM of each primer, 2.0 mM MgCl_2 , 200 mM dNTPs, 2 U Taq DNA polymerase and $10 \times$ buffer supplied with the enzyme. All reagents were from Fermentas, except for the primers, which were from Eurogentec. The PCR program comprised of an initial denaturation of 10 min at 95°C , followed by 35 cycles of 30 s at 94°C , 30 s annealing at 66°C , 1 minute 30 s elongation at 72°C , followed by a final extension of 2 min at 72°C [7]. The variants were detected by enzymatic digestion with MboI. Homozygotes and heterozygotes were discriminated by separation of the digested fragments in 3% agarose gel electrophoresis. The sizes of the wild type and mutated alleles were as follows: wild type 18-83A allele: 250 bp fragment; mutated 18-83G allele: 171 and 79 bp fragments.

The size of the PCR and digested products were checked by electrophoresis in 3% agarose gel stained with 10 mg/ml ethidium bromide (fig. 1) [7]. 83A/G-REN genotypes. Lane 1,2,4,5,8: heterozygotes 83A/83G fragments of 250, 171 and 79 bp; lane 3,6,7,9: negative 83A/83A fragments of 250 bp; lane 10: pBR HaeIII digest DNA molecular marker.

All medical assessments were performed on the first day of hospitalization of congestive heart failure patients before starting specific treatment. All investigations were also made for 40 healthy subjects.

The local institutional Ethics Committee approved the study and all participants gave their written informed consent.

Statistical analysis was carried out using SPSS for Windows 16.0 and MedCalc 10.3.0.0 software programs. The analysis of the differences between qualitative variables was performed by using the χ^2 test. Mean differences among continuous qualitative variables were evaluated with the Student's t-test (unpaired and paired), while nonparametric tests (Mann-Whitney U) were used to assess distribution variables that did not comply with normal conditions. A value of $P < 0.05$ was deemed statistically significant.

RESULTS

The characteristics of the patients and the subjects without cardiovascular disease are summarized in Table I. Heart failure etiology was ischemic in 60.46 % of patients. The average value of NT-pro BNP was $2991.24 \pm 2034.6 \text{ pg/ml}$ in accordance to ESC guideline criteria from 2008, for a certain diagnosis of chronic heart failure [5]. As it was expected, heart failure patients presented low lipid fraction values: Cholesterol = $162.36 \pm 38.28 \text{ mg/dl}$, LDL-Co = $104.88 \pm 27.60 \text{ mg/dl}$, Triglycerides = $109.12 \pm 55.84 \text{ mg/dl}$, HDL-Co = $35.68 \pm 9.55 \text{ mg/dl}$.

A/G renin genetic polymorphism distribution in heart failure patients with pathogenic potential was of 60.46% (homozygote form 4.65% and heterozygote form 55.81% as it is shown in figure 2). Distribution of REN A/G genetic mutation in heart failure patients with hypertension, obesity and diabetes mellitus is presented in Table II. The mean age in the control group was 48.33 ± 10.24 years. There were 32.5% men and 67.5% women. None of them presented the homozygote form of REN A/G mutation.

In return, the heterozygote form was detected in 37.5% of control patients who all were diagnosed with different grades of obesity or overweight. We determined $\chi^2(2.616)$ and p value (0.106), which show no statistically significant difference between REN A/G mutation in patients with chronic heart failure and those without chronic heart failure. We obtained the same result by using the Z- test: two samples for means with a p value of 0.124.

Figure 1. Agarose gel electrophoresis.

Figure 2. REN A/G mutation in heart failure patients and in control group.

Table I
Main features of heart failure patients vs control group

Variables	Heart failure patients (43 patients)	Control group (40 patients)	P
Age [years]	64.96 ± 13.19	48.33 ± 10.24	P < 0.0001
Males [%]	69.76%	32.5%	P = 0.0015
Smokers [%]	16.27%	45%	P = 0.009
BMI [kg/m ²]	26.60 ± 5.53	22.07 ± 4.89	P = 0.0002
Glycemia [mg/dl]	109.7 ± 45.01	100.9 ± 11.73	P = NS
Total Cholesterol [mg/dl]	162.36 ± 38.28	199.68 ± 22.59	P < 0.0001
LDL-Cholesterol [mg/dl]	104.88 ± 27.60	121.5 ± 9.77	P = 0.0005
HDL-Cholesterol [mg/dl]	35.68 ± 9.55	45 ± 6.33	P < 0.0001
Triglycerides [mg/dl]	109.12 ± 55.84	165.09 ± 32.12	P < 0.0001
Hypertension [%]	60.46%	-	-
Diabetes [%]	20.9 %	-	-
Nt-proBNP [pg/ml]	2991.24 ± 2034.6	-	-
Ejection fraction [%]	42.38 ± 8.35%	-	-

Table II

Genetic mutation polymorphism of REN A/G and hypertension, overweight or obesity and diabetes in heart failure patients

Condition (number of patients)	Hypertension (26)	Obesity and overweight (26)	Diabetes (9)
Negative AA %, (n)	61.53 (16)	44.11 (15)	77.77 (7)
Homozygote GG% (n)	0	5.88 (2)	22.22 (2)
Heterozygote AG%, (n)	38.46 (10)	50 (17)	0

Renin-angiotensin inhibitors therapy is undoubtedly of first choice in patients with heart failure. This therapy was administered to 81.39% of them, as follows: 69.76% received angiotensin-converting enzyme inhibitors (ACEI) and 11.62% received angiotensin receptor blockers (ARB's). 73.07% of patients with REN A/G pathogenic mutations were treated with ACEI and ARB's were prescribed only to 5.2% of them.

DISCUSSION

Despite all complex existing treatments, chronic heart failure remains a threatening public health issue. It is estimated that in developed countries 1-2% of adults suffer from chronic heart failure. More so, the percentage exceeds 10% in population over 70 years [8]. The association between high plasma levels of renin and cardiovascular disease was described for the first time in 1972 when Brunner *et al.* [9] showed a direct relationship between circulating renin, myocardial infarction and stroke event risk in patients with hypertension. During the following years, the involvement of renin in cardiovascular disease pathogenesis was extensively studied. In 1997, Alderman *et al.* [10] studied 1717 hypertensive subjects and had the same findings as Brunner, except for the correlation between high renin levels and stroke events. Framingham Heart study reported that high renin value increases general mortality in the first 3 years after the admission of 3298 patients with or without hypertension. However, this research observed no correlation between renin levels and ischemic heart disease [11]. Still, the authors do not exclude the possible implication of excessive renin secretion in the appearance of subclinical ischemic heart disease [11]. A high number of studies have associated renin with hypertension pathogenesis due to its decisive role in the renin-angiotensin-aldosterone system [RAS] functionality. Renin cuts the glycosylated form of angiotensinogen into angiotensin I [12], which in turn is converted into angiotensin II by angiotensin-converting enzyme [ACE] and kinase proteins. Angiotensin II has a great influence on hypertension and ischemic heart disease pathogenesis by exerting its main effects

via different types of receptors, especially AT1. Current research has shown that high plasma levels of renin increase general risk of mortality in patients diagnosed with chronic heart failure [13-15]. This important risk was also observed in asymptomatic patients with left ventricular dysfunction [16]. In addition, high circulating renin may be responsible for the development and enhancement of heart failure. Undoubtedly, the whole renin-angiotensin-aldosterone system is involved.

Therefore, current studies try to unravel the exact mechanisms by which each component of RAS system reaches high plasma levels in patients diagnosed with cardiovascular disease, diabetes mellitus or metabolic syndrome [12]. According to these findings, it has been established that genetic mutations lead to an excessive renin secretion or to AT receptors structure modifications. Renin genetic mutations have been previously studied, especially in patients with hypertension and less in subjects with heart failure. Actually, its gene (REN) plays a crucial role in the pathogenesis of essential hypertension and cardiovascular disease [17]. Most studies have focused on highlighting the A allele of single nucleotide polymorphisms (SNP) rs66939554 and rs6696954A [18] and also the 10607A and 12057T mutations. However, new studies are required in order to have relevant data related to renin genetic mutations in heart failure patients.

Our study showed that REN genetic mutations were found in 60.46% of heart failure patients in comparison with 37.5% of control subjects, this difference being without any statistical significance. The heart failure lot contained 38.46% patients suffering from hypertension and renin gene polymorphism. Interestingly, there was a big number [55.88%] of obese and overweight heart failure patients who presented renin gene polymorphism. Moreover, in all control subjects with obesity REN gene mutations were found. To date, no data concerning the association between high levels of renin and obesity has been reported, although further investigations are needed to define its in-depth mechanisms. A possible explanation would be that excessive renin secretion may determine extensive angiotensinogen release, which in turn increases the angiotensin I and II levels. Angiotensin II may have a key role to play in the

conversion of preadipocytes into adipocytes [19]. Angiotensin-converting enzyme (ACE) was found in the adipose tissue, too [20]. In this study 20.9% of patients suffered from both heart failure and diabetes mellitus. Concerning this issue, studies speculate that renin-angiotensin-aldosterone system increases insulin resistance [21]. As shown above, all patients diagnosed with heart failure had significantly high levels of NT-proBNP [over 2000 pg/ml], in accordance with ESC guide [4]. A recent study performed in Japan (2011) by Nishiyama *et al.* observed a strong and direct correlation between BNP and renin levels in patients with dilatative cardiomyopathy and heart failure. Furthermore, they suggested that those two parameters interact physiologically [22]. As we were expecting, heart failure patients presented low lipid fraction values. It is postulated that low lipid fraction levels suggest a bad prognosis for end-stage heart failure patients and at the same time they represent strong risk factors for mortality in these individuals [23–26]. Congestive hepatopathy occurs in an advanced chronic heart failure and leads to low plasma lipid fraction levels. It is also noted that intestinal endotoxins may translocate the swollen mucosa and cause the release of cytokines. The endotoxin or lipopolysaccharide is a toxic component of gram-negative bacterial wall which typically consists of a lipid A and a O polysaccharide. As it is well known, inflammation is as a key player in chronic heart failure which involves a variety of proinflammatory cytokines such as TNF α , IL-6, IL-1 and soluble receptors of TNF α - sTNFR-I and sTNFR-II. Plasma lipoproteins exert specific and selective effects on endotoxins by binding and neutralizing them. In this way the inflammatory cascade is blocked. Thus, low lipid fraction values are direct associated with end-stage heart failure,

which may be easily explained. According to the ESC guidelines [4, 8], ACEI and ARB's were administered to most of the heart failure patients, chiefly to those with REN genetic polymorphisms. Most studies reported that finding REN genetic polymorphisms in subjects without cardiovascular disease might signify a high mortality risk factor [11, 17, 27].

CONCLUSION

Although REN genetic polymorphisms was much more frequently found in patients with heart failure compared to healthy subjects, the current study could not demonstrate the involvement of A/G renin polymorphisms in the pathogenesis of congestive heart failure. More research including a larger number of patients with this pathology is required.

List of abbreviations:

- ACE: angiotensin II- converting anzyme
- AT 1: angiotensin receptor 1
- ESC: European Society of Cardiology
- HDL-CO: high-density lipoprotein cholesterol
- IL-6: interleukin-6
- IL-1: interleukin-1
- LDL-Co: low-density lipoprotein cholesterol
- NT-proBNP: N-terminal prohormone of brain natriuretic peptide
- NYHA class: New York Heart Association class
- RAS: renin-angiotensin-aldosterone system
- TG: triglycerides
- TNF α : tumor necrosis factor- alpha
- sTNFR-I and II: soluble receptors of TNF α

Ipoteze. Polimorfismul genetic al sistemului renina-angiotensină-aldosteron este implicat în patogeneza hipertensiunii arteriale, a bolii ischemice și a insuficienței cardiace (IC). Scopul studiului a fost analizarea polimorfismului genetic A/G a reninei la pacienții cu insuficiență cardiacă.

Material și metodă. A fost investigat polimorfismul genetic (utilizând metoda PCR) la 83 de pacienți internați în secția de Cardiologie a Spitalului de Recuperare, Cluj-Napoca, România. 43 de pacienți au fost diagnosticați cu insuficiență cardiacă (clasa NYHA III-IV), iar 40 de pacienți nu prezentau afecțiuni cardiovasculare (grup control). A fost evaluată prezența factorilor de risc cardiovascular și a fost determinat NT-proBNP. Rezultate: etiologia IC a fost ischemică în 60,46% dintre cazuri. Valoarea medie a NT-proBNP a fost $2991,24 \pm 2034,6$ pg/ml. Așa cum era de așteptat, pacienții cu insuficiență cardiacă au prezentat nivele mai scăzute ale lipidelor serice: colesterol total = $162,36 \pm 38,28$ mg/dl, LDL-colesterol = $104,88 \pm 27,60$ mg/dl, trigliceride = $109,12 \pm 55,84$ mg/dl, HDL-colesterol =

35,68 ± 9,55 mg/dl. A/G renin genetic polimorfism [cu potențial patogen] la pacienții cu insuficiență cardiacă a fost întâlnit la 60,46% dintre pacienți (homozigoți 4,65% and heterozigoți 55,81%). Mutațiile patologice au fost întâlnite doar la 38,46% din pacienții hipertensivi, dar la 55,88% dintre cei obezi/supraponderali și 22,22% dintre cei diabetici. Forma heterozigotă a fost prezentă la 37,5% din subiecții din grupul control.

Concluzie. Studiul de față nu reliefează implicarea polimorfismului A/G a reninei în patogeniza insuficienței cardiace.

Cuvinte cheie: polimorfism genetic, renina, insuficiență cardiacă.

Acknowledgements. This paper was published under the frame of European Social Fund, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/138776”.

The authors would like to thank all the participating members in the study. No conflict of interest was declared.

Corresponding author: Sitar-Tăut Adela-Viviana, C-tin Brâncuși Street No 181/5,
Cluj-Napoca, Romania; Phone 00400745461244,
E-mail adelasitar@yahoo.com

REFERENCES

1. NGUYEN G, MULLER DN. *The biology of the (pro) renin receptor*. J Am Soc Nephrol. 2010; 21(1):18-23.
2. SCHALEKAMP MA, DERKX FH, DEINUM J, DANSER AJ. *Newly developed renin and prorenin assays and the clinical evaluation of renin inhibitors*. J Hypertens. 2008; 26(5):928-937. Review.
3. VOLPE M, BATTISTONI A, CHIN D, RUBATTU S, TOCCI G. *Renin as a biomarker of cardiovascular disease in clinical practice*. Nutr Metab Cardiovasc Dis. 2012; 22 (4):312-317.
4. DICKSTEIN K, COHEN-SOLAL A, FILIPPATOS G, MCMURRAY JJ, PONIKOWSKI P, POOLE-WILSON PA, et al. *ESC guidelines for the diagnosis and treatment of acute and chronic heart failure 2008*. Eur Heart J. 2008; 29:2388-2442.
5. World Health Organization. *Obesity: preventing and managing the global epidemic. Report of a WHO consultation*. World Health Organ Technical Report Series 2000; 894: i-xii,1-253.
6. RUPERT JL, KIDD KK, NORMAN LE, MONSALVE MV, HOCHACHKA PW, DEVINE DV. *Genetic polymorphisms in the renin-angiotensin system in high altitude and low altitude native American populations*. Ann Hum Genet 2003; 67: 17-25.
7. PROCOPCIUC LM, CARACOSTEA G, ZAHARIE G, PUSCAS M, IORDACHE G, POPA M, et al. *Maternal/newborn genotype contribution of the renin-angiotensin system [Met235Thr, Thr174Met, I/D-ACE, A2350G-ACE, A1166C-AT2R1, C3123A- AT2R2, 83A/G- REN] to the risk of pre-eclampsia: a Romanian study*. J Renin Angiotensin Aldosterone Syst. 2011; 12(4):539-548.
8. McMURRAY JJ, ADAMOPOULOS S, ANKER SD, AURICCHIO A, BÖHM M, DICKSTEIN K, et al. *ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association [HFA] of the ESC*. EHJ 2012;33; 1787-1847.
9. BRUNNER HR, LARAGH JH, BAER L, NEWTON MA, GOODWIN FT, KRAKOFF LR, et al. *Essential hypertension: renin and aldosterone, heart attack and stroke*. N Engl J Med 1972; 286: 441-449.
10. ALDERMAN MH, MADHAVAN S, OOI WL, COHEN H, SEALEY JE, LARAGH JH. *Association of the renin-sodium profile with the risk of myocardial infarction in patients with hypertension*. N Engl J Med 1991; 324:1098-1104.
11. PARIKH NI, GONA P, LARSON MG, WANG TJ, NEWTON-CHEH C, LEVY D, et al. *Plasma renin and risk of cardiovascular disease and mortality: the Framingham Heart Study*. Eur Heart J 2007; 28:2644-2652.
12. POP DANA. *Sistemul Renină-Angiotensină-Aldosteron în patogeniza bolilor cardiovasculare*. Editura Clusium, Cluj-Napoca, 2007.
13. ALDI S, MARINO A, TOMITA K, CORTI F, ANAND R, OLSON KE, MARCUS AJ, LEVI R. *E-NTPDase1/CD39 modulates renin release from heart mast cells during ischemia/reperfusion: a novel cardioprotective role*. FASEB J. 2014 Oct 15. pii: fj.14-261867.
14. SIM JJ, SHI J, AL-MOOMEN R, BEHAYAA H, KALANTAR-ZADEH K, JACOBSEN SJ. *Plasma Renin activity and its association with ischemic heart disease, congestive heart failure, and cerebrovascular disease in a large hypertensive cohort*. J Clin Hypertens (Greenwich). 2014; 16(11):805-813.
15. SEVER PS, CHANG CL, PRESCOTT MF, GUPTA A, POULTER NR, WHITEHOUSE A, SCANLON M. *Is plasma rennin activity a biomarker for the prediction of renal and cardiovascular outcomes in treated hypertensive patients? Observations from the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT)*. Eur Heart J. 2012; 33(23):2970-2979.
16. BENEDICT CR, SHELTON B, JOHNSTONE DE, FRANCIS G, GREENBERG B, KONSTAM M, et al. *Prognostic significance of plasma norepinephrine in patients with asymptomatic left ventricular dysfunction*. Circulation 1996; 94:690-697.

17. VALDEZ-VELAZQUEZ LL, MENDOZA-CARRERA F, PEREZ-PARRA SA, RODARTE-HURTADO K, SANDOVAL-RAMIREZ L, MONTOYA-FUENTES H, *et al.* *Renin gene haplotype diversity and linkage disequilibrium in two Mexican and one German population samples.* J Renin Angiotensin Aldosterone Syst. 2011; 12(3):231-237.
18. SUN B, WILLIAMS JS, POJOGA L, CHAMARTHI B, LASKY-SU J, RABY BA, *et al.* *Renin gene polymorphism: its relationship to hypertension, renin levels and vascular responses.* J ReninAngiotensin Aldosterone Syst. 2011; 12 (4):564-571.
19. SCHLING P, LÖFFLER G. *Effects of angiotensin II on adipose conversion and expression of genes of the renin-angiotensin system in human preadipocytes.* Horm Metab Res. 2001; 33(4):189-95.
20. BARTON M, CARMONA R, ORTMANN J, KRIEGER JE, TRAUPE T. *Obesity-associated activation of angiotensin and endothelin in the cardiovascular system.* Int J Biochem Cell Biol. 2003; 35(6):826-837.
21. GEORGE BAKRIS. *Are There Effects of Renin–Angiotensin System Antagonists Beyond Blood Pressure Control?* American Journal of Cardiology. 2010; (105):21A-29A.
22. NISHIYAMA K, TSUTAMOTO T, KAWAHARA C, YAMAJI M, SAKAI H, YAMAMOTO T, *et al.* *Relationship between biological variation in B-type natriuretic peptide and plasma rennin concentration in stable outpatients with dilated cardiomyopathy.* Circ J. 2011; 75(8):1897-1904.
23. VREDEVOR DL, WOO MA, DOERING LV, BRECHT ML, HAMILTON MA, FONAROW GC. *Skin test anergy in advanced chronic heart failure secondary to either ischemic or idiopathic dilated cardiomyopathy.* Am J Cardiol. 1998; 82: 323-328.
24. RAUCHHAUS M, KOLOCZEK V, HANS-DIETER V, KEMP M, NIEBAUER J, FRANCIS DP, *et al.* *Inflammatory cytokines and the possible immunological role for lipoproteins in chronic heart failure.* Int J Cardiol. 2000; 76: 125-133.
25. RAUCHHAUS M, COATS AJS, ANKER SD. *The endotoxin-lipoprotein hypothesis.* Lancet. 2000; 356: 930-933.
26. RAUCHHAUS M, CLARK AL, DOEHNER W, DAVOS C, BOLGER A, SHARMA R, *et al.* *The relationship between cholesterol and survival in patients with chronic heart failure.* J Am Coll Cardiol.2003; 42:1933-1940.
27. WANG TJ, GONA P, LARSON MG, TOFLER GH, LEVY D, NEWTON-CHEH C, *et al.* *Multiple biomarkers for the prediction of first major cardiovascular events and death.* N Engl J Med 2006; 355:2631-2639.

Received February 10, 2015