

RELATIONSHIP OF HIGH CIRCULATING CYSTATIN C TO BIOCHEMICAL MARKERS OF BONE TURNOVER AND BONE MINERAL DENSITY IN ELDERLY MALES WITH A CHRONIC HEART FAILURE

POVEZANOST POVEĆANOG CIRKULIŠUĆEG CISTATINA C I BIOHEMIJSKIH MARKERA METABOLIZMA KOSTI I MINERALNE KOŠTANE GUSTINE KOD STARIJIH MUŠKARACA SA HRONIČNOM SRČANOM INSUFICIJENCIJOM

Biljana Bozic Nedeljkovic^{1,2}, Goran Loncar³, Tjasa Vizin⁴,
Zoran Radojicic⁵, Vera Popovic Brkic⁶, Janko Kos^{4,7}

¹Faculty of Biology, University of Belgrade, Serbia

²Institute for Medical Research, Military Medical Academy, Belgrade, Serbia

³Clinical Hospital Zvezdara, Belgrade, Serbia

⁴Faculty of Pharmacy, University of Ljubljana, Slovenia

⁵Institute of Statistics, Faculty of Organizational Sciences, University of Belgrade, Serbia

⁶Faculty of Medicine, University of Belgrade, Serbia

⁷Department of Biotechnology, Jozef Stefan Institute, Ljubljana, Slovenia

Summary

Background: The aim of the study was to investigate the association of Cystatin C (CysC) to biochemical markers of bone turnover and bone mass, and to evaluate its prognostic significance in elderly males with chronic heart failure (CHF).

Methods: A prospective cohort study was executed on sixty-eight males (mean age 68 ± 7 years) with mild to moderate CHF, together with 19 of corresponding age- and body mass index-matched healthy individuals who underwent cardiovascular, bone mineral density (BMD), and body composition assessment. Biochemical assessment of all subjects included NT-pro-BNP, parathyroid hormone (PTH), 25-hydroxy vitamin D (25(OH)D), CysC, and biochemical markers of bone turnover including osteocalcin (OC), alkaline phosphatase (ALP), β -CrossLaps (β -CTX), osteoprotegerin (OPG), and receptor activator of nuclear factor κ B ligand (RANKL).

Results: Serum CysC was significantly increased in males with CHF in comparison to healthy control ones. A significant positive association was found between CysC levels and OC in males with CHF, while OC and β -CTX increased in increasing CysC tertiles. In multivariate regression analysis,

Kratka sadržaj

Uvod: Cilj studije je bio da se ispita povezanost cistatina C (CisC) i biohemijskih markera metabolizma kosti i mineralne koštane gustine, kao i da se proceni prognostički značaj CisC kod starijih muškaraca sa hroničnom srčanom insuficijencijom (HSI).

Metode: Ovo istraživanje je prospektivna studija kohorte koju je činilo šezdeset osam muškaraca (prosečne starosti 68 ± 7 godina života) sa blagom do umerenom HSI i 19 zdravih osoba odgovarajuće starosti i indeksa telesne mase. Svi ispitanici su podvrgnuti proceni kardiovaskularnog sistema, mineralne koštane gustine i telesnog sastava. Biohemijska merenja kod svih ispitanika su uključivala određivanje NT-pro-BNP, paratiroidnog hormona (PTH), 25-hidroksi vitamina D (25 (OH) D), CisC i biohemijskih markera metabolizma kosti, uključujući osteokalcin (OC), alkalnu fosfatazu (ALP), beta-CrossLaps (β -CTX), osteoprotegerin (OPG) i ligand za receptor aktivator nuklearnog faktora B (RANKL).

Rezultati: Serumski CisC je značajno povećan u muškaraca sa HSI u poređenju sa nivoom kod zdravih osoba. Značajna pozitivna povezanost detektovana je između nivoa CisC i OC kod muškaraca sa HSI, i vrednosti OC i β -CTX su povećane kod pacijenata u rastućim CisC tercijarima. U multivarijantnoj regresionoj analizi je pokazano da su OC i pušenje

Address for correspondence:

Prof. Biljana Bozic Nedeljkovic, PhD
University of Belgrade, Faculty of Biology
Studentski trg 16, 11000 Belgrade, Serbia
Phone/Fax: +381 11 303 2356
e-mail: biljana@bio.bg.ac.rs, najbiljana@yahoo.com

OC and smoking were a significant determinant of CysC in males with CHF. Level of CysC was found to be positively associated with an increased fatal risk in males with CHF.

Conclusions: Serum osteocalcin is an independent predictor of CysC level in elderly males with CHF. Higher CysC level showed a negative relation to survival and bone loss in males with CHF. Further research is needed to confirm the potential role of CysC in the crosstalk between heart, kidney, bone, and energy metabolism in CHF.

Keywords: Cystatin C, osteocalcin, bone turnover markers, bone mineral density, chronic heart failure

Introduction

Chronic heart failure (CHF) and chronic kidney injury are two common chronic conditions in elderly adults that due to the association with morbidity/mortality are gaining increasing importance in healthcare system (1). Investigations directed to elucidating common pathological mechanisms are necessary.

It is well established that CHF is a complex interplay among genetic, neurohormonal, biochemical, and inflammatory changes (2). The interaction between heart and renal dysfunction has been an area of considerable interest of research in recent years. It is assumed that CHF is a condition characterized by the association of chronic inflammatory process in the heart and impairment of the renal function. In regard of that trend the term »cardiorenal syndrome« has been proposed to outline the interplay between simultaneous heart and renal dysfunctions, and to analyse the biochemical changes, which have influenced both heart and kidney functions (3).

Cystatin C (CysC) is a small protein molecule belonging to cysteine protease inhibitors family with its main biological role being extracellular inhibition of cathepsins. CysC is freely filtered by the renal glomerulus and then reabsorbed and fully catabolized by proximal renal tubules, afterwards excreted into the bloodstream without creating a complex with other secreted proteins in the blood. It is a good endogenous biomarker of kidney function/glomerular filtration ratio (4–6). Furthermore, it was found to be associated with heart extracellular matrix remodelling. The activated oxidants in the failing heart induce elevated CysC production from cardiomyocytes (7). Increased CysC level is related to a variety of cardiovascular pathological conditions that have influence on renal function (8–10). Higher levels of CysC were associated with an increased overall/cardiovascular cause of mortality in the population based cohort of ambulatory elderly persons (11). The CysC concentration is additionally an independent risk factor for heart failure in older adults (12). Therefore, serum CysC may be a useful surrogate marker of cardiovascular pathology. However, there are many unclear

značajne determinante nivoa CisC kod muškaraca sa HSI. Nivo CisC je u pozitivnoj korelaciji sa povećanim rizikom za mortalitet kod muškaraca sa HSI.

Zaključak: Serumski osteokalcin je nezavisan prediktor nivoa CisC kod starijih muškaraca sa HSI. Povećan nivo CisC je pokazao negativnu povezanost sa preživljavanjem i gubitkom kosti kod muškaraca sa HSI. Potrebno je da buduća istraživanja potvrde potencijalnu ulogu CisC u komunikaciji između srca, bubrega, kosti i energetskog metabolizma u hroničnoj srčanoj insuficijenciji.

Ključne reči: Cistatin C, osteokalcin, marker metabolizma kosti, mineralna koštana gustina, hronična srčana insuficijencija

facts about CysC as a marker for heart failure and its prognostic value in CHF. It is not clear which factors influence serum CysC values and there is not sufficient literature data about correlation of CysC values with other pathological processes associated with CHF, such as bone loss and metabolic syndrome.

Osteoporosis is the additional multifactorial pathological condition in elderly adults associated with poor quality of life and morbidity/mortality (13). Patients with heart failure have been reported to have lower bone mineral density, in part related to lower vitamin D and have higher frailty rates (14). The majority of available data indicates that the disturbances in bone turnover and the degree of bone loss are only subtle in CHF patients (15). There are data that CysC could be a promising biomarker for identification of elderly adults at high risk of hip fracture. Higher serum CysC is associated with an increased risk of hip fracture in elderly postmenopausal white females (16), but there is a paucity of data for males (17).

Consistent to our research aims in previous papers, we would like to continue investigation of crosstalk in cardiovascular-bone axis in CHF. The aim of our prospective investigation was to investigate the associations of CysC as the confirming biomarker of renal dysfunction, with biochemical markers of bone turnover and mass in a group of elderly males with CHF. Additionally, we intended to analyze the determinants of increased CysC in CHF and to evaluate the prognostic power of CysC for CHF and for six-year mortality in CHF patients.

Materials and Methods

Study population

The study population of this longitudinal prospective cohort study, which included 68 elderly male patients with CHF, has previously been characterized in detail by inclusion and exclusion criteria (18, 19). The control group consisted of 19 healthy subjects (HS), who did not take any medications. No previous medical illness was reported. Written informed consent for recruitment into the study and for follow-up for ten

years from recruitment was obtained from all subjects. The study was conducted according to the principles outlined in the Declaration of Helsinki and was approved by the Ethics Committee of the Clinical Medical Centre Zvezdara. All-cause mortality was evaluated after six years. The data on patients' deaths was obtained by phone contact with their relatives.

Clinical/cardiovascular assessment

All CHF males were categorized according to the New York Heart Association (NYHA) criteria (20). A physical exam was performed to assess CHF stability. Six-minute walk test (21), two-dimensional Doppler echocardiographic examination (22), and grip strength

determination (23) were measured according to standard protocols. Bone mineral density at lumbar spine (L1-L4) was estimated by using dual-energy-X-ray absorptiometry machine (Lunar prodigy advance, USA), according to standard protocol.

Laboratory analysis

Basal blood samples were taken at 8 a.m. from an antecubital vein within two consecutive months (March–April 2007). Serum samples were immediately deep frozen and kept at -70°C until assay. All molecules in blood samples were measured by the appropriate method given with detailed protocol in previous papers (18, 19).

Table I Baseline characteristics of elderly males with CHF and healthy subjects.

	Males with CHF (n = 68)	Healthy subjects (n = 19)	p
Age (years)	68 ± 7	67 ± 8	NS
BMI (kg/m ²)	27 ± 5	28 ± 3	NS
Waist (cm)	98 ± 13	100 ± 7	NS
Waist/hip ratio	1.02 ± 0.04	1.01 ± 0.06	NS
NYHA functional class			
NYHA class II, n (%)	51 (75)		
NYHA class III, n (%)	17 (25)		
Clinical histories			
Duration of CHF (years)	5 ± 4		
Smoking former / active, n (%)	18 (27) / 9 (13)	3 (16) / 5 (26)	
Ischemic / idiopathic dilatated CMP, n (%)	56 (82) / 12 (18)		
Mean blood pressure (mmHg)	101 ± 12	104 ± 7	NS
Hypertension, n (%)	43 (63)		
Atrial fibrillation, n (%)	20 (29)		
Pacemaker, n (%)	7 (10)		
History of myocardial infarction, n (%)	43 (63)		
Echocardiography			
LVEDD (mm)	66 ± 9	49 ± 4	<0.0001***
LVEF (%)	29 ± 8	65 ± 5	<0.0001***
E/Em ratio	21 ± 11	9 ± 3	<0.0001***
Physical performance			
Six-minute walking distance (m)	406 ± 84	583 ± 62	<0.0001***
Grip strength (kg)	94 ± 20	115 ± 18	<0.0001***
Body composition			
BMD L1-4 (g/cm ²)	1.094 ± 0.179	1.225 ± 0.231	0.010*
BMD L1-4 (T score)	-1.03 ± 1.49	0.08 ± 1.89	0.008**
BMD hip (g/cm ²)	0.880 ± 0.157	0.984 ± 0.184	0.015*
BMD hip (T score)	-1.41 ± 1.25	-0.58 ± 1.48	0.017*
Fat mass	21 ± 9	25 ± 7	0.052
Lean mass	54 ± 8	57 ± 4	0.011*

Data are expressed as mean ± SD or absolute number (percentage)

*** p < 0.0001, ** p < 0.01, * p < 0.05 elderly males with CHF vs. healthy subjects

BMD=bone mineral density, BMI=body mass index, CMP=cardiomyopathy, E/Em=transmitral flow velocity of E wave/mitral annular velocity at septal side, LVEF=left ventricular ejection fraction, LVEDD=left ventricular end-diastolic diameter, NYHA=New York Heart Association

Cystatin C determination

The levels of CysC in the stored deep frozen serum samples of patients and HS were measured by specific sandwich-type ELISA, developed at the Department of Biochemistry and Molecular Biology, Jo ef Stefan Institute and Krka, as described (24). A microtiter plate was coated with a rabbit-affinity purified anti-CysC polyclonal antibody. Recombinant human CysC was used for preparation of calibration curves. Sera from patients were diluted 1: 100 prior to being applied to the walls of microtiter plate. As a detection antibody, 1A2 monoclonal antibody was used, conjugated to horseradish peroxidase. A substrate for peroxidase was 3,3', 5,5'-tetramethylbenzidine liquid substrate system (TMB, Sigma). The reaction was stopped by addition of 2 mol/L H₂SO₄, and the absorbance was measured at 450 nm on mini-reader (TECAN, Sunrise). The measured values of diluted samples were compared with the calibration curve and expressed in ng/mL of serum.

Clinical/biohumoral characteristics

The general baseline characteristics of CHF and HS are presented in Table I. The elderly males with

CHF had lower left ventricular ejection fraction, together with six-minute walking distance, grip strength, lean mass, and BMD, but greater LVEDD and E/Em ratio.

As shown in Table II, males with CHF had significantly higher levels of NT-pro-BNP, PTH, OPG, RANKL, ALP, OC, β -CTx, and CysC, while 25(OH) D levels were lower compared to HS.

Statistical analysis

Descriptive statistics were presented as mean values with standard deviation or median with interquartile range for numeric variables, or as absolute numbers with percentages for categorical variables. Evaluation of normality was performed by Kolmogorov-Smirnov test. Student t-test was used to calculate differences between mean values. Mann-Whitney U-test was used to determine differences between median values. χ^2 -test tested differences in frequencies between the studied groups. Comparisons across three groups were done with ANOVA (group means). The Pearson coefficient was used for linear correlation between variables. Finally, since variables are inter-related, multivariate regression

Table II Baseline values of biochemical parameters in elderly males with CHF and healthy subjects.

	Males with CHF (n = 68)	Healthy subjects (n = 19)	p value	
Clearance creatinine (mL/min)	63 \pm 22	80 \pm 13	0.002 **	
Creatinine (μ mol/L)	119.6 \pm 28.6	99.7 \pm 12.9	0.004 **	
BUN (mmol/L)	8.5 \pm 3.8	6.3 \pm 1.6	0.019 *	
Uric acid (μ mol/L)	414 \pm 106	371 \pm 192	NS	
Urin Ca (mmol/L)	2.3 \pm 1.5	3.7 \pm 2.4	0.004 **	
hsCRP (mg/L)	5.40 \pm 9.74	2.87 \pm 3.77	NS	
Serum albumin (g/L)	43 \pm 3	45 \pm 3	NS	
Glucose (mmol/L)	5.3 \pm 0.6	5.6 \pm 0.5	NS	
Cholesterol (mmol/L)	5.5 \pm 1.1	5.9 \pm 0.9	NS	Mean \pm SD
Triglyceridi (mmol/L)	1.6 \pm 0.6	1.7 \pm 1.2	NS	
HDL (mmol/L)	1.28 \pm 0.34	1.45 \pm 0.42	NS	
LDL (mmol/L)	3.54 \pm 0.94	3.76 \pm 0.80	NS	
25(OH)D (nmol/L)	31.9 \pm 14.8	48.9 \pm 22.0	<0.0001 ***	
PTH (pg/mL)	78.3 \pm 33.8	39.9 \pm 11.7	<0.0001 ***	
ALP (U/L)	81.5 \pm 28.4	67.7 \pm 19.2	0.018 *	
OC (ng/ml)	38.3 \pm 19.1	29.2 \pm 7.7	0.047 *	
β -CTx (pg/mL)	517 \pm 278	375 \pm 159	0.036 *	
Cystatin C (pg/mL)	1354 \pm 427	1125 \pm 301	0.031 *	
NT-pro-BNP (pg/mL)	1809 (2742)	67 (74)	<0.0001 ***	
OPG (pg/mL)	79.8 (121.7)	10.8 (63.5)	<0.0001 ***	Me \pm IQR
RANKL (pg/mL)	130.9 (125.8)	47.8 (44.4)	<0.0001 ***	

Data are expressed as mean \pm standard deviation (Mean \pm SD) or median \pm interquartile range (Me \pm IQR)

***p < 0.0001, **p < 0.01, *p < 0.05, elderly males with CHF vs. healthy subjects

ALP=alkaline phosphatase, BUN=blood urea nitrogen, 25(OH) D=25-hydroxy vitamin D, NT-pro-BNP=N-terminal pro-brain natriuretic peptide, OPG=osteoprotegerin, OC=osteocalcin, β -CTx= β -CrossLaps, HDL=High Density Lipoprotein, LDL=Low Density Lipoprotein, PTH=parathyroid hormone, sRANKL=serum receptor activator of nuclear factor κ B ligand

analysis, stepwise method, was performed to assess the independent variables that may explain CysC variability. The probability of F was used to select the variables to be included in the model, the variables with p-values less than 0.05 were entered and variables with p-values larger than 0.10 were removed from the model. A $p < 0.05$ was considered to indicate statistical significance. Statistical analysis was performed using the SPSS software (Chicago, IL).

Results

CysC level in elderly CHF males

The elderly males with CHF had significantly higher serum CysC level than HS (Table II). Additionally, we demonstrated that elderly males with CHF NYHAIII had significantly higher CysC levels compared to HS (1433 ± 99 vs. 1125 ± 301 pg/mL, $p = 0.014$).

Further, ROC analysis was performed to evaluate sensitivity and specificity of CysC between the groups studied. The area under the ROC curve for CysC levels was 0.659 (95%CI 0.537–0.785), $p < 0.034$ (Figure 1). It was a low but statistically significant for CHF discrimination in comparison to HS. The calculated P/N ratio for CysC levels was 1250 pg/mL (sensitivity 0.42; specificity 0.58).

Relations between CysC with clinical/ biohumoral characteristics

Correlation analysis of CysC with various clinical/biohumoral parameters in CHF and HS are shown in Table III.

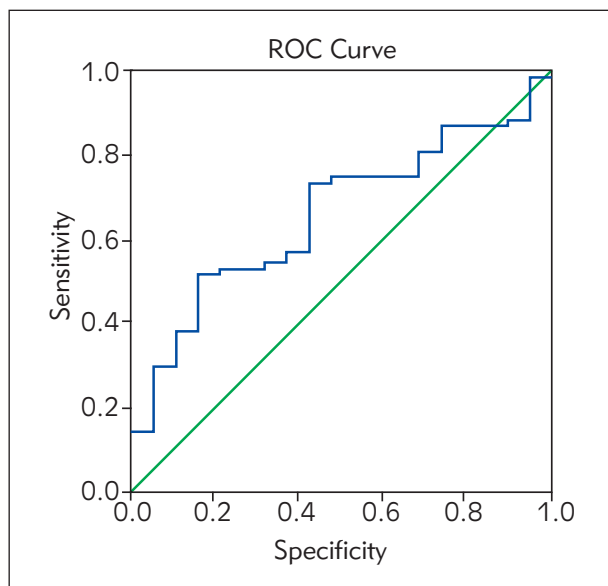


Figure 1 Receiver operator characteristic (ROC) curves for detection of chronic systolic heart failure in elderly males by CysC.

In HS CysC level was positively associated with age, BMD, and with tendency to statistical significance of cholesterol and OPG levels, while it was negatively associated with clearance of creatinine, six-minute walk test, lean mass and PTH (with tendency to statistical significance). All presented associations in HS were non-existent in CHF. On the other hand, in CHF CysC level was positively associated with mean blood pressure and levels of cholesterol, LDL and OC, which was not evident in HS.

Although, in CHF the association between CysC and age was not detected, the potential impact of age and heart failure on CysC levels was further evaluated. Figure II presents CysC levels in CHF and HS, grouped according to age tertiles. This analysis demonstrated that serum CysC levels were significantly higher in CHF in T1 and T2 age tertiles compared to HS in the same age tertiles. However, there was no difference in CysC levels between CHF and HS at the highest age tertiles (T3). In addition, HS in T3 had a significantly increased CysC levels compared to HS in T1 age tertiles.

Table III Association of CysC values with biochemical and clinical variables in elderly males with CHF and healthy subjects.

	Cystatin C	
	Healthy subjects	CHF
Age		
r (p)	0.666 (0.002)	NS
Smoking		
r (p)	NS	0.335 (0.005)
Clearance creatinine		
r (p)	-0.615 (0.005)	NS
Cholesterol		
r (p)	0.446 (0.056)	0.269 (0.027)
LDL		
r (p)	NS	0.240 (0.048)
Mean blood pressure		
r (p)	NS	0.291 (0.016)
Six-minute		
r (p)	-0.517 (0.024)	NS
Lean mass		
r (p)	-0.530 (0.020)	NS
BMD L1-L4 T g/cm ²		
r (p)	0.538 (0.017)	NS
BMD L1-L4 T score		
r (p)	0.539 (0.017)	NS
PTH		
r (p)	-0.452 (0.052)	NS
OPG (log-transformed)		
r (p)	0.454 (0.051)	NS
OC		
r (p)	NS	0.289 (0.017)

BMP=Bone Mineral Density; LDL=Low Density Lipoprotein; OPG=Osteoprotegerin; OC=Osteocalcin; PTH=Parathyroid Hormone

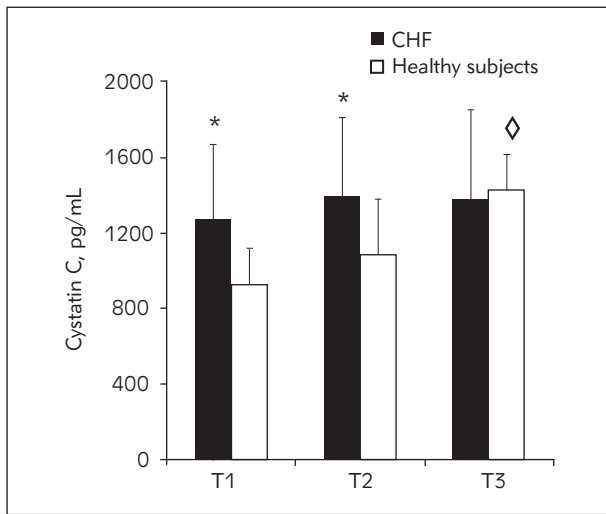


Figure 2 Serum CysC levels in elderly males with CHF and healthy subjects according to age tertiles.

(T1=lowest to T3=highest)

Data are expressed as mean ± standard deviation (Mean ± SD)

*p<0.05, CHF vs. healthy subjects

◇p<0.05, healthy subjects in T3 tertiles vs. T1 tertiles

Biochemical markers of bone turnover in elderly CHF males according to CysC tertiles

Levels of OC and β-CTx were significantly higher in the highest CysC tertiles compared to middle CysC tertiles in CHF, and for OC they appeared only in T1 (Figure III). ALP values in T3 were also significantly higher in comparison to T2 (87.2±28.9 vs. 68.4±20.2 U/L, p=0.015).

Moreover, LVEF, LVEDD, and E/Em ratios were significantly impaired in the higher CysC tertiles. Levels of cholesterol and LDL were increased in the higher compared to lower CysC tertiles (Figure III).

Multivariate logistic regression analysis

A stepwise forward logistic regression analysis was performed. Odds ratio (OR), 95% confidence intervals (95%CI) and Nagelkerke's R squared (R²) are presented for the last stage (step 3) (Table IV). Independent association with CysC levels, in CHF, was noted for smoking and OC, which together had an R² of 0.435. Other included variables (age, CHF duration, BNP, Cr, glucose, cholesterol, LDL, HDL, ALP,

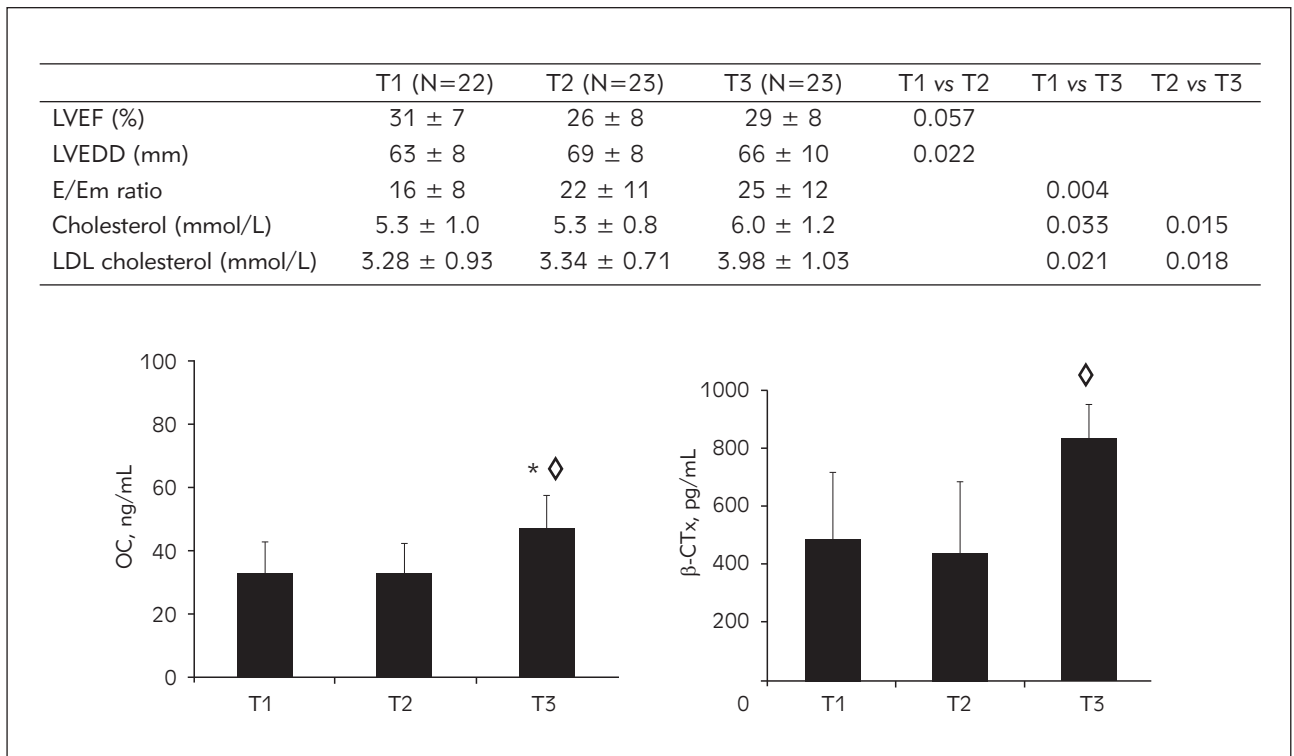


Figure 3 Some cardiovascular and metabolic parameters and serum osteocalcin and β Cross-Laps levels in elderly males with CHF according to CysC tertiles.

(T1=lowest to T3=highest)

Data are expressed as mean ± standard deviation (Mean ± SD)

*p<0.05, T3 vs. T1; ◇p<0.05, T3 vs. T2

LVEF=Left Ventricular Ejection Fraction; LVEDD=Left Ventricular End-Diastolic Diameter; E/Em=transmitral flow velocity of E wave/ mitral annular motion velocity at septal side, LDL=Low Density Lipoprotein, OC=osteocalcin; b-CTx=b Cross-Laps

β -CT α , PTH, 25(OH) D, OPG, RANKL) showed a statistically insignificant contribution to the model. After finding that smoking influences the level of CysC in CHF we compared the level of CysC between smoking and non-smoking males with CHF. The smoking males with CHF had lower CysC level compared to non-smoking subjects with CHF (1104 \pm 485 vs. 1469 \pm 385 pg/mL, $p=0.018$).

Mortality of elderly CHF males according to CysC tertiles

Survival curve for mortality according to CysC tertiles is presented in Figure IV. Levels of CysC were found to be positively associated with an increased risk of mortality in CHF (log-rank test, $p>0.05$). The elderly males with CHF in the highest CysC tertiles had a statistically significantly lower survival period

Table IV Multivariate regression analysis with CysC as dependent variable.

CHF patients	B	p	F (p)
Cystatin C (Constant)	684.393		
Smoking	186.446	0.007	7.458
Osteocalcin	5.717	0.027	(<0.0001)
$R^2 = 0.435$			

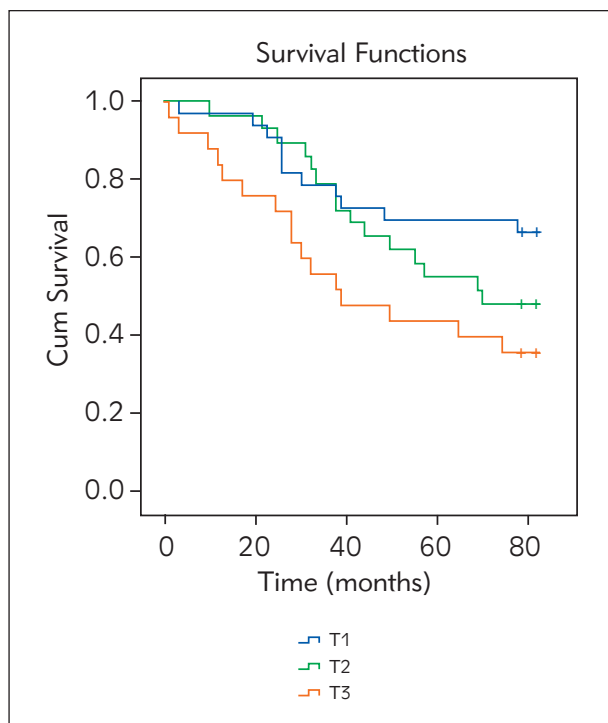


Figure 4 Survival curves of elderly males with CHF according to CysC tertiles

compared to males with CHF in lower CysC tertiles. Additionally, we used Cox regression analysis for detection of mortality rate of elderly males with CHF grouped according to CysC values adjusted for age and ClCr in the tertiles. Results of this analysis after adjustment confirmed that CHF in T3 had shorter survival period compared to T1 ($p=0.005$).

Discussion

As far as we are aware, this is the first report of relation of bone turnover to levels of CysC in elderly males with CHF. Our main findings are: 1) elderly males with CHF in the highest CysC tertiles have increased bone turnover (measured by level of bone-derived substances); 2) increased OC is an independent determinant of CysC in elderly males with CHF; 3) elderly males with CHF and higher CysC values had a decreased survival rate during the six-year-period, emphasising CysC as the prognostic marker of outcome in CHF. These findings are in line with the recent publication that bone-derived substances (25), some of which have hormonal properties, are related to CHF. Thus a new light was shed on the bone-cardiovascular axis. All these findings support the hypothesis that CysC and OC may play a significant role in crosstalk between heart, bone, and kidney in CHF.

CysC is a marker of renal function (5) and cardiovascular disease risk (11, 12), but its precise role in CHF has not been well established. CysC is associated with both more advanced left ventricular diastolic dysfunction and right ventricular systolic dysfunction; therefore it remains an independent predictor of long-term prognosis in chronic systolic (26). In line with previous studies we confirmed that CysC levels were increased among elderly males with CHF in comparison to HS. There was a significant relative risk of systolic CHF in elderly males associated with elevated CysC above the calculated cut-off of 1250 pg/mL. In agreement with previous studies presented in literature about the influence of CysC on CHF (11, 12), CHF patients in the T1 and T2 age tertiles had higher CysC values compared to HS in the same age tertiles. Additional confirmation of this finding was a significantly increased CysC level in elderly males with CHF NYHAIII compared to HS, while elderly males with CHF NYHAII had no statistically significantly increased CysC values in the same comparison. On the contrary; there is no difference in CysC values between CHF and HS in the highest age tertiles. The level of CysC in the highest age tertiles in HS was significantly higher compared to the lowest age tertiles. Apparently, this result is in contrast to literature data. It was reported that CysC production rate is not dependent on sex, age, body weight, or diet (27), but is altered by factors like inflammation, smoking status, corticosteroid treatment, and CRP level (28). Since the HSs in this study had normal renal function, did not use corticosteroids, and did not present ele-

vated CRP levels, we may assume that smoking status and some other processes that have not been investigated, which are related to healthy ageing, had an influence on CysC level in the oldest HS.

The level of CysC is greatly influenced by thyroid function (29, 30). The thyroid hormones increase CysC *in vivo* and stimulate CysC production in bone cells *in vitro* (31). Firstly, an increased serum PTH was independently associated with various well-established parameters of heart failure progression, endothelial dysfunction, neuroendocrine activation (NT-pro-BNP, adiponectin) and could potentially be used to monitor progression of CHF (32, 33). Although, PTH did not correlate with CysC and there were no differences in PTH levels among males with CHF in different CysC tertiles, we may assume that PTH alterations might be responsible for the increased levels of CysC in CHF. This hypothesis is supported by an increased bone turnover in the highest CysC tertiles. Secondly, body fat is a significant determinant of CysC. The highest CysC level was detected in the group of obese healthy young males (34). The obesity should not influence the increased CysC in our CHF patients since all subjects were BMI-matched and there were no differences in the total fat mass. Nevertheless, 31% of CHF males were obese, while 20% HS were obese. Visceral obesity was present in 59% of HF versus 50% in controls (19). Due to these facts the potential influence of fat on the increasing levels of CysC in CHF cannot be excluded.

In the next part of study, we analyzed the association of CysC with other parameters. In HS we detected correlation between CysC and age. It is in agreement with higher CysC in older men (>59 years) compared to middle-aged men (<59 years) (35). On the other hand, the association of CysC with age in our HS is contrary to literature data that CysC is not influenced by aging (27). The significant finding of this part of investigation is that all the associations of CysC detected in HS were not detected in CHF. Contrary to that, new associations in CHF were detected. These findings support the hypothesis that production of CysC is impaired and influenced by a lot of factors in CHF, which may change its primary role in health. The negative associations of CysC with physical performance expressed by six-minute walk and lean mass were noted in HSs in this study. This finding may be explained by the detected age-related CysC elevation and age-related decrease in skeletal muscle mass, which is associated with functional limitation, disability, falls, and with a spectrum of various consequences (36, 37). However, our results are in contrast to the study of Baxmann and colleagues who did not detect any correlation between lean mass and CysC in healthy males and females (38). These inconsistencies in CysC association with peripheral lean mass and physical performance may be attributed to differences in the studied groups. The patients in the study of Baxmann and colleagues were significantly younger, with different

physical activities, and it was a mixed cohort of men and women. In elderly males with CHF these associations between CysC and physical performance were not noticed. The reason for it might be a muscle alteration induced by CHF, although our patients were non-cachectic. This might bear responsibility for the reduction in functional performance in this cohort. It is in accordance with the claim that physical inactivity may be important in terms of peripheral muscle mass reduction (39). The positive correlation of CysC and BMD in our HS might be explained by facts that CysC affects the BMP signalling cascades in osteoblastic cells and then promotes osteoblast differentiation, mineralization, and bone formation (40). This association was not detected in CHF males, thus indicating an impaired production of CysC in CHF. Additionally, our finding on positive correlation between CysC and OPG in HS is in accordance with the previously published paper of Kulcsar-Jakab and colleagues (35). This correlation was not found in CHF males.

Currently, there is a growing interest in the potential roles of CysC and OC in the metabolic syndrome, the regulation of glucose and fat metabolism. The association of cardiovascular disease and metabolic syndrome is well established (41). CysC level in CHF males positively associates with mean blood pressure and level of cholesterol and LDL as well. These associations do not show in HS. This is in line with results which suggest that CysC may affect metabolic factors adversely thus contributing to the development of the metabolic syndrome (42). Results of this study may help to explain the link between CysC, metabolic changes (higher mean blood pressure, level of cholesterol and LDL) and the development of heart failure in elderly males. Further, we had shown that CysC positively associates with OC in CHF males. This newly detected association between CysC and OC in CHF was interesting for further analysis/investigation. This finding supports the involvement of both molecules, CysC and OC, in pathogenesis of heart failure in elderly population and related changes such as metabolic changes/syndrome and bone loss. Low OC levels are strongly associated with the metabolic syndrome in an elderly population (43). This is in agreement with our results that CHF males without metabolic syndrome presented the increased OC levels. Furthermore, it is in line with the finding of negative correlation between OC and fat mass in elderly males (44). Linking this with the result that CHF males with the highest CysC had lower survival period compared to patients with lower CysC, and finding that OC is an independent determinant of CysC in CHF, we may assume that CysC and OC may have important roles, linking heart failure with bone metabolism, metabolic regulation, and energy homeostasis. Future studies are welcome in order to find a potential molecular link between these variables in CHF.

Study limitations

Several issues should be considered in the interpretation of our data. The principal limitation of our study is the relatively small sample size, which makes it impossible to draw firm conclusions in regard to hard endpoint. Secondly, our study was not originally designed to test the relation of CysC to CHF. Accordingly, it did not include information on other markers, which limits our insight into the mechanisms underlying the observed associations and interactions. Thirdly, the wide confidence intervals of the selected associations indicate a limited statistical strength. Thus, our study may lack precision in estimates of the observed associations and interactions, which suggests the need to replicate these findings in larger and other population samples in order to verify the strength of the observed associations. Finally, our results apply to a population of elderly CHF males without metabolic syndrome, diabetes, and cachexia. Thus, they are unlikely to apply to another CHF subpopulation.

In conclusion, the level of CysC was elevated in elderly CHF males and had adverse influence on the survival of these subjects. Increased CysC in CHF without the influence of metabolic syndrome, diabetes, and cachexia is independently determined by the increased OC. Our findings may indicate a cross-sectional metabolic association of increased CysC with increased level of bone turnover markers, espe-

cially OC, and reduced bone mass in non-cachectic, non-diabetic, non-metabolic syndrome elderly CHF males. This bone-derived substance, OC, which influences CysC in CHF, might place the bone at the centre of cardiovascular disease associated with chronic kidney disease. Most importantly, factors that indicate the inter-regulation of these substances in bone, heart, kidney, and other tissues such as fat and muscles, and subsequent secretion into the circulation, have not been investigated. They could provide entirely new avenues for therapeutic intervention. Future studies are welcome to evaluate the predictive values of CysC, to perform evaluation for clinical outcomes as well as for beneficial potential of CysC or OC suppression in CHF.

Acknowledgments. This work was supported by Serbian grant 175033, Slovenian grant P46811 and Bilateral Project between Slovenia and Serbia (principal investigators JK and BBN)

The abstract was presented at the 7th Conference on Experimental and Translational Oncology (2013, Slovenia).

Conflict of interest statement

The authors state that they have no conflicts of interest regarding the publication of this article.

References

- Leibowitz D. Left ventricular hypertrophy and chronic renal insufficiency in the elderly. *Cardiorenal Med* 2014; 4: 168–75.
- Braunwald E. Biomarkers in heart failure. *N Engl J Med* 2008; 358: 2148–59.
- Tripodskiadis F, Starling RC, Boudoulas H et al. The cardiorenal syndrome in heart failure: cardiac? renal syndrome? *Heart Fail Rev* 2012; 17: 355–66.
- Coll E, Botey A, Alvarez L et al. Serum cystatin C as a new marker for noninvasive estimation of glomerular filtration rate and as a marker for early renal impairment. *Am J Kidney Dis* 2000; 36: 29–34.
- Fliser D, Ritz E. Serum cystatin C concentration as a marker of renal dysfunction in the elderly. *Am J Kidney Dis* 2001; 37: 79–83.
- National Kidney Foundation Chronic Kidney Disease Guidelines. [(Accessed 4 January 2008)]. http://www.kidney.org/professionals/KDOQI/guidelines_ckd/toc.htm
- Xie L, Terrand J, Xu B et al. Cystatin C increases in cardiac injury: a role an extracellular matrix protein modulation. *Cardiovasc Res* 2010; 87: 628–35.
- Moran A, Katz R, Smith NL et al. Cystatin C concentration as a predictor of systolic and diastolic heart failure. *J Card Fail* 2008; 14: 19–26.
- Ix JH, Shlipak MG, Chertow GM et al. Cystatin C, left ventricular hypertrophy, and diastolic dysfunction: data from the Heart and Soul Study. *J Card Fail* 2006; 12: 601–7.
- Nosaka, Nakamura K, Kusano K et al. Cystatin C indicates diastolic dysfunction. *Congest Heart Fail* 2013; 19: E35–E39.
- Shlipak MG, Sarnak MJ, Katz R et al. Cystatin C and the risk of death and cardiovascular events among elderly persons. *New Engl J Med* 2005; 352: 2049–60.
- Marković D, Jevtović-Stoimenov R, Čosić V, Stošić B, Dinić V, Marković-Živković B, Janković JR. Clinical utility of survivin (BIRC5), novel cardiac biomarker, as a prognostic tool compared to high-sensitivity c-reactive protein, heart-type fatty acid binding protein and revised lee score in elderly patients scheduled for major non-cardiac surgery: a prospective pilot study. *J Med Biochem* 2018; 37: 110–20.
- Prevention and management of osteoporosis. *World Health Organ Tech Rep Ser.* 2003; 921: 1–164. back cover
- Tarighi S, Najafi M, Hossein-Nezhad A, Ghaedi H, Meshkani R, Moradi N, Fadaei R, Kazerouni F, Shanaki M. Association between two common polymorphisms of vitamin D binding protein and the risk of coronary artery disease: A case-control study. *J Med Biochem* 2017; 36: 349–57.

15. Zittermann A, Schleithoff SS, Koerfer R. Markers of bone metabolism in congestive heart failure. *Clin Chim Acta* 2006; 366: 27–36.
16. Fried LF, Biggs ML, Shlipak MG et al. Association of kidney function with incident hip fracture in older adults. *J Am Soc Nephrol* 2007; 18: 282–6.
17. Ensrud KE, Parimi N, Fink HA et al. Osteoporotic Fractures in Men Study Group. Estimated GFR and risk of hip fracture in older men: comparison of associations using cystatin C and creatinine. *Am J Kidney Dis* 2014; 63: 31–9.
18. Bozic B, Loncar G, Prodanovic N et al. Relationship Between High Circulating Adiponectin With Bone Mineral Density And Bone Metabolism In Elderly Males With Chronic Heart Failure. *J Card Fail* 2010; 16: 301–7.
19. Loncar G, Bozic B, von Haehling S et al. Association of adiponectin with peripheral muscle status in elderly patients with heart failure. *Eur J Intern Med* 2013; 24: 818–23.
20. The Criteria Committee of the New York Heart Association Criteria for diagnosis and treatment of heart failure. Little, Brown and Company, 1994.
21. ATS statement: guidelines for the six-minute walk test. ATS Committee on Proficiency Standards for Clinical Pulmonary Function Laboratories. *Am J Respir Crit Care Med* 2002; 166: 111–17.
22. Silvestry FE, Kerber RE, Brook MM et al. Echocardiography-guided interventions. *J Am Soc Echocardiogr* 2009; 22: 213–31.
23. Massy-Westropp N, Rankin W, Ahern M et al. Measuring grip strength in normal adults: Reference ranges and a comparison of electronic and hydraulic instruments. *J Hand Surg Am* 2004; 29: 514–9.
24. Kos J, Stabuc B, Schweiger A et al. Cathepsins B, H, and L and their inhibitors stefin A and cystatin C in sera of melanoma patients. *Clin Canc Res* 1997; 3: 1815–22.
25. Vervloet MG, Massy ZA, Brandenburg VM et al; CKD-MBD Working Group of ERA-EDTA Bone: a new endocrine organ at the heart of chronic kidney disease and mineral and bone disorders. *Lancet Diabetes Endocrinol* 2014; 2: 427–36.
26. Tang WH, Van Lente F, Shrestha K et al. Impact of myocardial function on cystatin C measurements in chronic systolic heart failure. *J Card Fail* 2008; 14: 394–9.
27. Newman DJ. Cystatin C. *Ann Clin Biochem* 2002; 39: 89–104.
28. Knight EL, Verhave JC, Spiegelman D, et al. Factors influencing serum cystatin C levels other than renal function and the impact on renal function measurement. *Kidney Int* 2004; 65: 1416–21.
29. Fricker M, Wiesli P, Brandle M, et al. Impact of thyroid dysfunction on serum cystatin C. *Kidney Int* 2003; 63: 1944–7.
30. Wiesli P, Schwegler B, Spinaz GA et al. Serum cystatin C is sensitive to small changes in thyroid function. *Clin Chim Acta* 2003; 338: 87–90.
31. Schmid C, Ghirlanda-Keller C, Zwimpfer C et al. Triiodothyronine stimulates cystatin C production in bone cells. *Biochem Biophys Res Commun* 2012; 419: 425–30.
32. Bozic B, Loncar G, Prodanovic N et al. Parathyroid hormone response to vitamin D insufficiency in elderly males with chronic heart failure. *Physiol Res* 2011; 60: S155–63.
33. Loncar G, Bozic B, Dimkovic S et al. Association of increased parathyroid hormone with neuroendocrine activation and endothelial dysfunction in elderly men with heart failure. *J Endocrinol Invest* 2011; 34: e78–85.
34. Chew-Harris JS, Florkowski CM, George PM et al. The relative effects of fat versus muscle mass on cystatin C and estimates of renal function in healthy young men. *Ann Clin Biochem* 2013; 50: 39–46.
35. Kulcsar-Jakab E, Petho Z, Pap Z et al. Cystatin C as a potential predictor of osteoprotegerin levels in healthy men, a cross-sectional, observational study. *BMC Musculoskelet Disord* 2015; 16: 227. doi: 10.1186/s12891-015-0684-1.
36. Baumgartner RN, Waters DL. Sarcopenia and sarcopenic obesity. In: Pathy MSJ, Sinclair AJ, Morley JE, editors. Principles and practice of geriatric medicine. West Sussex, England: JohnWiley & Sons Ltd.; 2006. p. 909–34.
37. von Haehling S, Morley JE, Anker SD. An overview of sarcopenia: facts and numbers on prevalence and clinical impact. *J Cachexia Sarcopenia Muscle* 2010; 2: 129–33.
38. Baxmann AC, Ahmed MS, Marques NC et al. Influence of muscle mass and physical activity on serum and urinary creatinine and serum cystatin C. *Clin J Am Soc Nephrol* 2008; 3: 348–54.
39. Mancini DM, Walter G, Reichel N et al. Contribution of skeletal muscle atrophy to exercise intolerance and altered muscle metabolism in heart failure. *Circulation* 1992; 85: 1364–73.
40. Danjo A, Yamaza T, Kido MA et al. Cystatin C stimulates the differentiation of mouse osteoblastic cells and bone formation. *Biochem Biophys Res Commun* 2007; 360: 199–204.
41. Emerging Risk Factors Collaboration, Wormser D, Kaptoge S et al. Separate and combined associations of body-mass index and abdominal adiposity with cardiovascular disease: collaborative analysis of 58 prospective studies. *Lancet* 2011; 377: 1085–95.
42. Magnusson M, Hedblad B, Engström G et al. High levels of cystatin C predict the metabolic syndrome: the prospective Malmö Diet and Cancer Study. *J Intern Med* 2013; 274: 192–9.
43. Oosterwerff MM, van Schoor NM, Lips P et al. Osteocalcin as a predictor of the metabolic syndrome in older persons: a population-based study. *Clin Endocrinol (Oxf)* 2013; 78: 242–7.
44. Kindblom JM, Ohlsson C, Ljunggren O et al. Plasma osteocalcin is inversely related to fat mass and plasma glucose in elderly Swedish men. *J Bone Miner Res* 2009; 24: 785–91.

Received: February 1, 2018

Accepted: March 17, 2018