

# IS CIRCULATING GLA-RICH PROTEIN LINKED WITH CORONARY CALCIUM AND CARDIOVASCULAR PATHOLOGY IN PATIENTS WITH ATRIAL FIBRILLATION OR HEART FAILURE? A PILOT STUDY

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## ABSTRACT

**INTRODUCTION:** Nowadays Gla-rich protein (GRP) is recognized as a novel biomarker playing a pivotal role in the crosstalk between chronic inflammation and vascular calcification.

**AIM:** The aim of this article is to study the link between circulating GRP, cardiovascular pathology, and the degree of arterial calcification evaluated by the coronary arterial calcium score (CACS) in a Bulgarian population sample.

**MATERIALS AND METHODS:** Adult participants (n=81) of both genders were divided into: controls (n=41)—subjects with estimated moderate-to-high risk without known cardiovascular diseases (CVDs) and a combined CVD group (n=40)—patients with paroxysmal or persistent atrial fibrillation in sinus rhythm, and heart failure subjects with preserved ejection fraction. A structured interview was carried out for evaluation of the classical CVD risk factors. CACS was determined by multislice computed tomography. Routine laboratory parameters were extracted from medical records. Serum levels of total GRP, matrix Gla protein, and osteocalcin were estimated by commercial ELISA kits. Standard statistical methods (descriptive statistics, Student's t-test and Spearman's correlation) were applied. Statistical significance was considered at  $p < 0.05$ .

**RESULTS:** Significantly lower GRP levels were established in patients with coronary calcium compared to those without calcium deposits. Clear tendency for decreased levels of GRP was observed in the combined CVD group vs controls. Circulating GRP significantly correlates with uncarboxylated matrix Gla protein. An association between serum GRP, CRP, and low-density lipoproteins (LDLs) was demonstrated.

**CONCLUSION:** This study adds new information regarding the role of circulating GRP as a new player in calcification inhibition. Our findings illuminate the link between total circulating GRP, CVD pathology, and the degree of coronary calcification.

**Keywords:** *Gla-rich protein (GRP), coronary arterial calcium score (CACS), atrial fibrillation, heart failure*

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## INTRODUCTION

As cardiovascular diseases (CVDs) are one of the major causes of death worldwide, a significant part of scientific research is focused on elucidating the molecular mechanisms underlying their development and progression. Vascular calcification (VC) is a process of calcium phosphate deposition in the vessel wall due to impaired balance between the regulatory mechanisms that contribute to the mineralization of the vessel wall and those that inhibit it (1). VC is a multifactorial process, but the loss of calcification inhibitors is a major mechanism for its enhancement (2,3).

Gla-rich protein (GRP) is a circulating vitamin K-dependent protein, also known as an upper zone of growth plate and cartilage matrix associated protein (UCMA) (4). A decade earlier GRP has been suggested to act as a negative regulator of osteogenic differentiation, a novel secretory cartilage-specific protein with implications in osteogenesis, a modulator of calcium availability in the extracellular matrix (ECM), and as a potential inhibitor of soft tissue calcification (4,5). In addition, it plays a role as an inhibitor of pathological VC and as an anti-inflammatory agent in the cardiovascular system (6,7). Nowadays GRP is recognized as a novel biomarker, which plays a pivotal role in the crosstalk between chronic inflammation and calcification in the articular, renal and cardiovascular systems (8).

Similarly to matrix Gla protein (MGP), another vitamin K-dependent protein, acting as local inhibitor of soft tissue calcification and vascular mineralization (9,10), GRP  $\gamma$ -carboxylation is essential for its calcification inhibitory function (8). In the study of Viegas et al. (6) it was demonstrated that GRP expression and accumulation are upregulated by a calcification process and possibly GRP is a component of the fetuin-A-MGP calcification inhibitory system. Moreover, it was demonstrated that GRP is included in the fetuin-A-MGP complex at the sites of valvular calcification. Also, only the  $\gamma$ -carboxylated GRP inhibits effectively calcification and osteochondrogenic differentiation through  $\alpha$ -smooth muscle actin upregulation and osteopontin downregulation. In the same *in vitro* study it was demonstrated that addition of carboxylated GRP is able to reduce both vascular calcification and proinflammatory responses

of articular and immune cells (6,8). GRP calcium-binding properties and its association to calcification processes indicate that its function might be associated with prevention of calcium-induced signaling pathways and direct mineral binding leading to blockage of crystal formation and maturation. High levels of GRP are found in sites of pathological calcification possibly due to its capability to bind calcium phosphate crystals directly (11). An impaired  $\gamma$ -carboxylation status of GRP is considered to be associated with pathological calcification, including vascular calcification, osteoarthritis, and certain cancers (11,12). It is considered that circulating GRP is a systemic inhibitor of ectopic calcification through its involvement in the inhibition of Ca/P mineral crystal formation and maturation, with profound consequences on vascular calcification (7).

The studies examining the role of circulating GRP as a novel marker of vascular calcification and inhibitor of inflammation are extremely scarce. To our knowledge, circulating GRP levels and their link to CVD have not examined in Bulgarian population.

## AIM

The aim of our pilot study was to examine the link between circulating GRP, CVD pathology and the degree of arterial calcification evaluated by the CAC score in Bulgarian population sample.

## MATERIALS AND METHODS

### *Patients and Methods*

The current study included 81 Bulgarian patients who were admitted to St. Marina University Hospital Varna between October 2018 and January 2020. In the control group were included 41 subjects who were estimated to be with moderate-to-high risk for CVD but without known CVD. The other 40 subjects formed the combined CVD group—23 patients with paroxysmal and persistent atrial fibrillation (AF) and 17 patients with heart failure (HF) with preserved ejection fraction (ejection fraction >40%, HFpEF) in sinus rhythm at the time of hospitalization. Information about age and history of CVD, smoking status, presence and treatment of arterial hypertension, hyperlipidemia, type 2 diabetes mellitus, and current use of anticoagulant medication were collected through a structured interview. Hypertension was defined as blood pressure

(BP) >140/90 mmHg at the time of examination or with a history of elevated BP and on antihypertensive medication. According to the cutoff values of ESC Guidelines, hyperlipidemia was defined as elevated total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) or triglycerides (TG) or by the use of lipid-lowering medications (13). All patients were physically examined for heart rate, blood pressure, weight, height, and waist circumference (WC). Body mass index (BMI) was calculated according to the standard formula. Patients with proven ischemic heart disease or stroke, cardiomyopathy, type 1 diabetes, chronic renal disease stage IV or more, known thyroid gland disease, and active cancer were excluded from the study.

The study was approved by the local Ethical Committee at the Medical University of Varna (Protocol No 75/ 07.06.2018). Written informed consent was obtained from all participants in the study.

#### **Coronary Artery Calcification Measurement**

All participants were examined on a multi-slice computed tomography in order to assess the presence of coronary artery calcification. CAC scans were performed on a Siemens Somatom Definition (Dual Source 2×64) CT scanner (Erlangen, Germany) using standardized imaging protocols and were interpreted by a single trained physician blinded to the clinical characteristics of the patients. Coronary artery calcification was defined as a lesion above 130 Hounsfield units, with an area equal to three pixels. CAC score (CACS) was calculated using the Agatston criteria (14). The presence of CAC was defined as an Agatston score >0 Agatston units (AU). According to the CACS results, the patients were classified into one of the following categories: CACS=0 AU (no coronary calcium), CACS>0 AU (presence of coronary calcium).

#### **Biochemical Measures**

Data for routine biochemical parameters, lipid profile parameters (TG, TC, HDL cholesterol, LDL cholesterol) and C-reactive protein (CRP) were extracted from the medical documentation. Fasting venous blood was drawn at admittance in the clinic. Blood samples were centrifuged at 1600xg for 15 min, and blood plasma and serum were separated and stored at -80°C until analysis of specific laboratory parameters. A commercial ELISA kits based on

specific competitive mono-antibodies were used for measuring the plasma levels of total GRP (Develop, China), circulating ucMGP (Cusabio, Wuhan, China), uncarboxylated (ucOC), and carboxylated osteocalcin (cOC) (BlueGene Biotech, Shanghai, China). The ratio between the concentrations of circulating ucOC and cOC (UCR) was calculated and used as an inverse indicator of vitamin K status. A lower OCR reflects good vitamin K status (15).

#### **Statistical Analysis**

Data are presented as mean±standard deviation, or number (n) and percentage. Statistical analysis was performed by GraphPad software version 7 (GraphPad Software, San Diego, CA USA) and SPSS v.23 (SPSS Inc., Chicago, IL, USA). Student's t-test was used to compare the means of two groups. Spearman correlation analysis was used for evaluation of the associations between the tested variables. Statistical significance was considered at  $p<0.05$ .

## **RESULTS**

Table 1 represents the baseline patients' characteristics. Significant differences between CVD and non-CVD patients were indicated for age, anthropometric measurements, AH duration, and smoking status. CACS for CVD group was three times higher compared to the non-CVD controls. Significantly higher values were indicated for serum glucose, creatinine, urea, and uric acid. More pronounced changes of the tested lipid parameters were found for LDL cholesterol.

We indicated a clear tendency for a decrease in circulating GRP levels for CVD patients vs. non-CVD controls (Fig. 1). Although non-significant, the same trend was preserved when the CVD participants were divided according to CVD pathology—lower GRP values were found for the HF group than those for AF patients ( $1.06\pm 0.8$  vs.  $1.29\pm 1.02$  ng/mL).

For assessing the changes in circulating GRP to the presence or absence of arterial calcium deposits, we stratified the patients according to their CACS. The data reveal a significantly lower circulating GRP (with 23.4%) in the group with coronary calcium (CACS>0) vs. patients without CAC (CACS=0) (Fig. 2).

To assess the link between circulating GRP and the tested risk factors for atherosclerosis we per-

Table 1. Patients' baseline characteristics

Parameter	Control Mean ± SD (n = 41)	CVD Patients Mean ± SD (n = 40)	P
Age (years)	53.39 ± 9.81	68.88 ± 7.03	0.001
Male gender (%)	39.0	32.5	0.352
BMI (kg/m <sup>2</sup> )	27.65 ± 5.66	30.36 ± 5.61	0.039
WC (cm)	92.00 ± 16.02	104.89 ± 12.91	0.001
AH duration (years)	6.11 ± 5.21	12.05 ± 8.02	0.001
Smoking duration (years)	17.92 ± 8.69	28.69 ± 12.66	0.013
Diabetes duration (years)	5.50 ± 3.54	12.93 ± 12.5	0.209
CACS (AU)	80.84 ± 285.45	353.17 ± 560.66	0.007
Glucose (mmol/L)	5.81 ± 0.82	7.44 ± 3.34	0.004
Urea (mmol/L)	5.83 ± 0.87	8.37 ± 4.99	0.050
Creatinine (mmol/L)	68.05 ± 13.50	83.24 ± 23.76	0.001
Uric acid (mmol/L)	318.28 ± 111.36	388.96 ± 99.51	0.009
Total cholesterol (mmol/L)	5.25 ± 1.01	4.86 ± 1.19	0.126
Triglycerides (mmol/L)	1.41 ± 1.04	1.47 ± 0.87	0.765
HDL cholesterol (mmol/L)	1.39 ± 0.41	1.44 ± 0.45	0.575
LDL cholesterol (mmol/L)	3.26 ± 0.76	2.79 ± 0.92	0.016
CRP (mg/L)	0.61 ± 0.45 9	5.54 ± 6.19	0.282
GRP (ng/mL)	1.11 ± 0.78	0.91 ± 0.77	0.080
ucMGP (µg/mL)	4.42 ± 3.37	5.69 ± 4.94	0.057

Abbreviations: BMI—body mass index; AH—arterial hypertension; CACS—coronary arterial calcium score; HDL—high-density lipoprotein; LDL—low-density lipoprotein; CRP—C-reactive protein; GRP—Gla-rich protein; ucMGP—uncarboxylated matrix Gla protein.

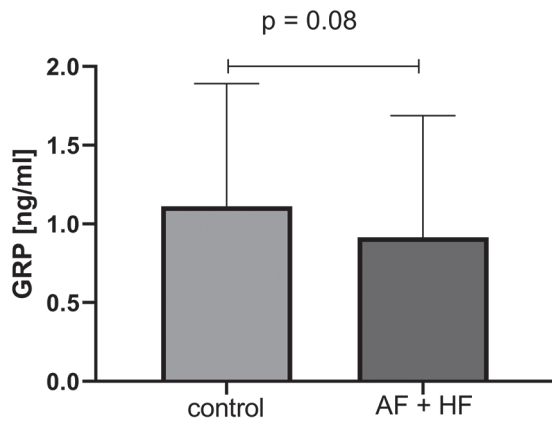


Fig. 1. Circulating serum GRP levels in controls (non-CVD patients) and in the combined CVD group (AF and HF patients).

Data are given as mean ±SD; statistical significance was considered at  $p < 0.05$ ; GRP—Gla-rich protein; CVD—cardiovascular disease; AF—atrial fibrillation; HF—heart failure with preserved ejection fraction.

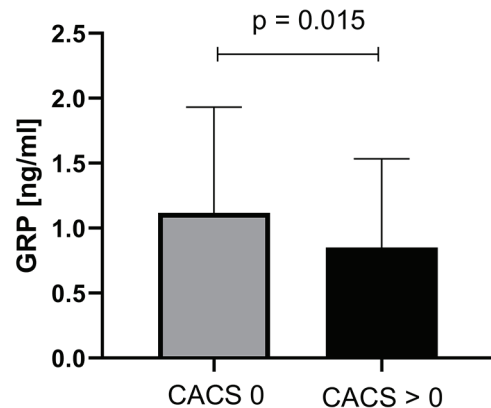


Fig. 2. Circulating serum GRP levels in patients without coronary calcium (CACS=0) and in participants with arterial calcium deposits (CACS>0).

Data are given as mean ±SD; statistical significance was considered at  $p < 0.05$ ; GRP—Gla-rich protein; CACS—coronary arterial calcium score.

formed Spearman correlation analysis for controls and CVD patients. Of the tested lipid parameters negative correlation with borderline significance was found for total cholesterol ( $r=-0.287$ ;  $p=0.056$ ) and triglyceride levels ( $r=-0.293$ ;  $p=0.051$ ) for the combined CVD group. For the same group a negative trend for a decrease in circulating GRP with CRP as a marker of inflammation ( $r=-0.187$ ;  $p=0.416$ ) was indicated. In the control non-CVD group we found significant negative associations only with smoking duration ( $r=-0.417$ ;  $p=0.0197$ ). Serum GRP tended to decrease with CACS for CVD and non-CVD patients. Circulating GRP correlated positively with circulating ucMGP for the control subjects ( $r=0.288$ ;  $p=0.05$ ) and for the combined CVD group ( $r=0.561$ ;  $p<0.0001$ ).

When the patients were divided according to their CACS, significant moderate association was found between circulating GRP and ucMGP and negative correlation with smoking status for both groups with or without coronary calcium. CRP, TC, TG and LDL-C tended to increase with the decrease of serum GRP (Table 2).

We did not find interrelations between circulating GRP, anthropometric parameters, duration of arterial hypertension, diabetes, both cOC and ucOC or OCR levels.

## DISCUSSION

Our study aimed to estimate the relationship between circulating total GRP with CVD pathology and vascular calcification. In CVD patients (combined AF + HF group), we have also examined the interaction between GRP, certain risk factors for CVD

and with the circulating levels of ucMGP as proven VC inhibitor.

We observed a tendency, although not significant, for a decrease in circulating levels of total GRP in the CVD patients, which is consistent with other studies (6,16,17). In the study of Viegas et al. (7), the levels of serum GRP sharply decrease in chronic kidney disease (CKD) patients with the severity of CKD pathology, e.g. from stage 2 CKD to stage 4 CKD. Our data also revealed lower serum GRP not only for the combined CVD group, but also for patients with more severe CVD pathology, such as heart failure.

Vascular calcification is a process under strict regulation of calcium and phosphate deposition in the intima and media layers of the vessel wall and in cardiac valves. The inhibition of mineral crystal formation is critical for prevention the destructive effects of calcium deposits and ectopic calcification. This process is actively inhibited by the functional calcification inhibitors, including MGP and fetuin-A (18). GRP is another constitutive component of the inhibitory system composed of MGP and fetuin-A preventing crystal formation and maturation (7). It is assumed that deficiency in GRP may result in increased mineral maturation promoting VC (7). In agreement with these data our study revealed significantly lower GRP levels in patients with coronary artery calcium (CACS>0) compared to those without coronary calcium (CACS=0).

In the present study, we found a positive association between circulating GRP and ucMGP in both CVD patients and in those with coronary calcium. To our knowledge, this is the first study examining the relation between these two inhibitory proteins of

*Table 2. Spearman correlation analysis between circulating GRP, tested risk factors for CVD and biochemical parameters in patients with or without coronary calcium deposits. Statistical significance was considered at  $p<0.05$ .*

Tested Parameter	CACS > 0	CACS = 0
	Spearman r; p	Spearman r; p
Smoking duration (years)	-0.389; p = 0.045	-0.386; p = 0.084
TC (mmol/L)	-0.189; p = 0.193	-0.048; p = 0.764
TG (mmol/L)	-0.148; p = 0.309	-0.108; p = 0.496
LDL-C (mmol/L)	-0.155; p = 0.293	-0.054; p = 0.733
CRP (mg/L)	-0.044; p = 0.871	-0.500; p = 0.267
ucMGP ( $\mu\text{g}/\text{mL}$ )	$r = 0.563$ ; $p \leq 0.0001$	0.441; $p=0.003$

Abbreviations: TC—total cholesterol; TG—triglycerides; LDL-C—low-density lipoprotein cholesterol; CRP—C-reactive protein; ucMGP—undercarboxylated matrix Gla protein

VC in the serum of CVD patients. It is not surprising that such positive relationship between circulating GRP and ucMGP as GRP is a component of a protein inhibitory complex (GRP–MGP–fetuin A) present at the sites of aortic valve calcification (7).

Our results demonstrate a clear trend for a decrease of total serum GRP with the increase of serum CRP for both CVD patients and in those with CACS >0. In addition, serum LDL levels also tend to increase with the decrease of circulating GRP. It is well known that calcification and inflammation are interconnected processes contributing to atherosclerosis development. There are data showing that decreased GRP levels are linked with increased pro-inflammatory responses of articular cells, and thus confirming the dual role of GRP—as calcification inhibitor and anti-inflammatory agent (19,20). Thus, it is considered that GRP may have strong implications in calcification and/or inflammation-related diseases such as CVD (6).

One of the limitations of the current study is the small number of studied patients. The GRP assay used for measuring circulating GRP cannot distinguish carboxylated and uncarboxylated GRP forms. Our study, for the first time, demonstrates a decrease in circulating total GRP in patients with CVD and in those with coronary calcium deposits. We have shown a significant relationship between circulating GRP and ucMGP confirming the notion that these two Gla proteins are components of a complex VC inhibitory system. An association between circulating GRP and serum CRP and LDL levels was demonstrated, further contributing to the hypothesis regarding the dual role of GRP as a calcification inhibitor and an anti-inflammatory agent. Our findings add new information regarding the role of circulating GRP as a new player in calcification inhibition.

## CONCLUSION

Our study illuminates the link between total circulating GRP and CVD pathology and the degree of coronary calcification. However, further research is needed to fully elucidate the molecular mechanisms of GRP action and to clarify its role as a potential novel valuable biomarker evaluating the development and the degree of cardiovascular risk and VC.

## Disclosure Statement:

The authors report no potential conflict of interest.

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