Matrix Gla-protein expression in peripheral blood mononuclear cells is related to risk factors in cardiovascular diseased patients

Periferik kan mononükleer hücrelerinde Matriks Gla-protein ekspresyonu, kardiyovasküler hastalarda risk faktörleri ile ilişkilidir

Abstract

Objectives: Matrix Gla protein (MGP) is a calcification inhibitor that plays a role in preventing soft tissue calcification and local mineralization of the vascular wall. The present study aimed to assess the expression of MGP in Peripheral Blood Mononuclear Cells (PBMC) in adult patients with CVD pathologies and its association with the presence and severity of coronary artery calcium score (CACS) and conventional CVD risk factors.

Methods: MGP expression was measured in 87 individuals using real time qPCR. Subgrouping was performed according etiologic and metabolic CVD risk factors.

Results: A clear trend for a decreased MGP expression was observed in all subgroups with high CVD risk. This decrease was significant in abdominally obese hypertensive individuals and in those with dyslipidemia. MGP expression was significantly lower in patients representing high Total cholesterol and LDL cholesterol levels. A positive correlation between MGP expression and smoking status in patients with coronary calcium and in the CVD group was established. Atrial hypertension duration correlated negatively with MGP expression in the group without coronary calcium deposits.

Conclusions: The current study supports the hypothesis that MGP expression in PBMC probably reflects CVD pathology and is related to lipid metabolism dysregulation.

Keywords: biomarkers; cardiovascular disease; gene expression; matrix Gla-protein; peripheral blood mononuclear cells.

Amaç: Matrix Gla proteini (MGP), yumuşak doku kalsifikasyonunu ve damar duvarının lokal mineralizasyonunu önlemede rol oynayan bir kalsifikasyon inhibitörüdür. Bu çalışma, KVH patolojileri olan erişkin hastalarda Periferik Kan Mononükleer Hücrelerinde (PBMC) MGP ekspresyonunun ve bunun koroner arter kalsiyum skoru (CACS) ve konvansiyonel KVH risk faktörlerinin varlığı ve şiddeti ile ilişkisini değerlendirildi.
Yöntem: MGP ifadesi, gerçek zamanlı qPCR kullanarak 87 kişiide ölçüldü. Etiyolojik ve metabolik KVH risk faktörlerine göre alt gruplandırma yapıldı.

Bulgular: Yüksek KVH riski olan tüm alt gruplarda azalmış MGP ekspresyonu için açık bir eğilim gözlemdi. Bu azalma abdominal obez hipertansiyon bireylerde ve dislipidemisi olanlarda anlaşılmıyordu. MGP ekspresyonu, yüksek Total kolesterol ve LDL kolesterol seviyelerini temsil eden hastalarda önemli ölçüde daha düşüktü. Koroner kalsiyumlu hastalarda ve KVH grubunda MGP ekspresyonu ile sigara içme durumu arasında pozitif bir ilişki saptanmıştır. Atrial hipertansiyon süresi, koroner kalsiyum birikimi olmayan grupta MGP ekspresyonu ile negatif korelasyon gösterdi.

Tartışma: Mevcut çalışma, PBMC'deki MGP ekspresyonunun muhtemelen CVD patolojisinin yansıtmayı ve lipid metabolizmasını düzenlemesi ile ilgili olduğu hipotezini desteklemektedir.

Anahtar kelimeler: biyobelirteçler; kardiyovasküler hastalıklar; Matrix Gla-proteini; gen ekspresyonu; periferik kan mononükleer hücreleri.

Introduction

Matrix Gla protein (MGP) is a local calcification inhibitor that plays essential role in preventing calcification of soft tissues and the vascular wall local mineralization [1]. It is secreted mainly by chondrocytes and vascular smooth muscle cells in the arterial tunica media [2]. In vascular endothelial cells it participates in early endothelial differentiation [3], and is important for preventing endothelial dysfunction that contributes to vascular calcification [4]. The importance of MGP as vascular calcification inhibitor has been demonstrated in a study on MGP-knock out mice which developed massive arterial calcification within 6–8 weeks after birth [5]. In another study, lack of MGP gene causes arteriovenous malformations in MGP–/– mice [3]. MGP protein requires posttranslational modifications for its activation – γ-glutamate carboxylation and serine phosphorylation. Thus, several MGP isoforms can exist: dephosphorylated-uncarboxylated (dp-ucMGP) – the fully inactive form, phosphorylated-uncarboxylated (p-ucMGP) and dephosphorylated-carboxylated (dp-cMGP) isoforms with partial activity, and as the most active form phosphorylated-carboxylated (p-cMGP) isoform [6]. Rat models revealed that both carboxylated and uncarboxylated MGP are also produced in aortic smooth muscle cells [7].

In cardiovascular disease (CVD) research, the focus is predominantly on studying the role of circulating MGP isoforms (γ-glutamate carboxylated and serine phosphorylated) as an inhibitors of vascular calcification [8, 9]. An association between total circulating MGP fraction and CVD risk factors but not with coronary artery calcification has been found in two independent studies on men and women with no clinically apparent CVD [10]. In a recent review Barrett and coauthors (2018) report a relationship between different serum MGP isof orm and vascular calcification in atherosclerotic CVD patients. Data they summarize are controversial and the conclusion is that this association is not completely elucidated, primarily due to a measurement of the total MGP content with no distinction of the different circulating MGP isoforms.

Data regarding the gene expression of MGP in clinical settings are scarce and usually on biopsy samples, an unpleasant and invasive manipulation. Another approach for testing MGP gene expression is by using easy accessible peripheral blood mononuclear cells (PBMC), representing the only site of active gene expression in blood. PBMC's expression profiling provides a potentially useful tool for search of biomarkers, reflecting system pathologies [11]. Multiple studies revealed alterations of mRNAs, microRNAs and epigenetic modification (methylation) profile in a variety of disorders leading to the conclusion that PBMC mimic conditions of some tissues which they contact and can be used as a non-invasive and suitable source of biomarkers [12]. PBMC metabolism has been studied in relation to both acute [13] and chronic [14] diseases, including CVD [15, 16]. To date, there are some isolated reports on MGP gene expression in PBMC. For example, it has been shown that MGP is synthesized and γ-carboxylated in human monocytes and T-lymphocytes and is involved in adaptive immune response [17]. Macrophage accumulation and infiltration is related to early plaque calcification in atherosclerosis which in turn gives evidence for the relation between PBMC and vascular calcification in CVD. In addition, inflammation activity, characterized by macrophage infiltration has been shown to precede the osteogenic transformation of vascular smooth muscle cells and the release of calcifying extracellular vesicles [18, 19]. To our knowledge, detailed studies on MGP expression in PBMC and its relationships to CVD pathology and risk factors are very scarce. The present prospective study aimed to assess the expression of MGP in PBMC in adult patients with CVD pathologies and its association with the presence and severity of coronary artery calcium score (CACS) and with conventional CVD risk factors.
Materials and methods

Patients

The research related to human use has complied with all the relevant national regulations, institutional polices, and in accordance with the tenets of the Helsinki Declaration, and has been approved by the author’s Institutional Review Board – Ethical Committee at the Medical University of Varna (Protocol No 75/07.06.2018). All participants provided written informed consent prior to participate in the study.

The current study included 87 Bulgarian patients (57 females and 30 males), admitted at the Cardiology Clinics, University Hospital – Varna between October 2018 and January 2020. The patients were divided into two groups – patients with CVD (n=46) and patients without known CVD but with estimated moderate to high risk for CVD (control group, n=41). The patients in CVD group (n=46) were split into two subgroups according to their CVD pathology – with paroxysmal or persistent atrial fibrillation (AF group, n=29) and with heart failure with preserved ejection fraction (ejection fraction >40%, HFrEF) who were in sinus rhythm at the time of hospitalization (HF group, n=17). The control group comprised 41 non-CVD patients. Age and history of CVD, presence of arterial hypertension, presence of hyperlipidemia, smoking status, type 2 diabetes mellitus presence and duration were assessed through a structured interview at admittance in the hospital.

Hypertension was defined as blood pressure (BP) >140/90 mm Hg at the time of examination or a history of elevated BP and intake of antihypertensive medication [20]. Hyperlipidemia was defined as elevated total cholesterol (TC), low density lipoprotein cholesterol (LDL-C) or triglycerides (TG) according to the ESC Guidelines cut-off values [21]. All patients were physically examined for blood pressure, weight, height, and waist circumference (WC). WC below 88 cm for females and below 102 cm for males was considered as normal; WC above 88 cm for women and above 102 cm for men was an indicator for abdominal obesity. Body mass index (BMI) was calculated using the formula weight/height² and expressed in kg/m². BMI below 24.9 kg/m² was considered as normal, BMI between 25.0 kg/m² and 29.9 kg/m² for overweight, and obesity was indicated for BMI above 30 kg/m². Patients with proven ischemic heart disease or stroke, type 1 diabetes mellitus, cardiomyopathy, known thyroid gland diseases, chronic renal disease (IV stage or more, with eGFR<30 mL/min/1.73 m²), and active cancer were excluded from the study.

Coronary artery calcium measurement

In order to assess the presence of coronary artery calcification, all participants underwent a multislice computed tomography examination. Coronary artery calcium scans were performed with a Siemens Somatom Definition (Dual Source 2×64) CT scanner using standardized imaging protocols. Thirty to forty consecutive tomographic slices were obtained at 3 mm intervals, 1 cm below the carina and progressing caudally to include the entire coronary tree. A single trained physician blinded to the clinical characteristics of the patients interpreted the scans. Coronary artery calcification was defined as a lesion of >130 Hounsfield units with an area equal to three pixels.

Coronary artery calcium score was calculated according the Agatston criteria [22]. The presence of coronary artery calcium was defined as an Agatston score >0 Agatston units (AU). Patients were classified into one of the following categories, according to the CACS results: CACS=0 AU (absence of coronary calcium, n=37), CACS<0 AU (n=49).

Biochemical parameters measurement

Routine laboratory parameters – glucose, urea, creatinine, uric acid (UA), total cholesterol (TC), triglycerides (TG), HDL-cholesterol (HDL-C), and LDL-cholesterol (LDL-C) for each patient were extracted from the medical documentation. These parameters were analyzed in fasting blood using biochemical analyzer (ADVIA 1800 Chemistry System, Germany) at the day of blood sampling. Castelli risk indexes (TC/HDL-C and LDL-C/HDL-C) and estimated glomerular filtration rate (eGFR) were calculated. Plasma levels of circulating uMGP were determined by a commercial ELISA kit (Cusabio, Wuhan, China).

Peripheral blood mononuclear cells collection and gene expression analysis

For the purpose of gene expression analyzes peripheral blood mononuclear cells (PBMC) were isolated from whole blood samples collected in lithium heparin vacutainer tubes. The separation of PBMC was carried out using Ficoll separation medium with density 1.077 g/L and LeucoSep™ centrifuge separation tubes (by GreinerBioOne, Austria) containing porous barrier which enable cell separation by means of density gradient centrifugation and purification following the manufacturer’s instructions.

From the collected PBMC RNA was extracted with Tri reagent (Ambion®, Life Technologies, USA). First strand cDNA synthesis was performed with 0.1 µg of total RNA using Thermo Scientific M-MuLV reverse transcriptase (USA) following the steps of manufacturer’s instructions.

Quantitative gene expression analysis was performed using two-step real-time qPCR. Each reaction was performed in triplicate and amplified in a reaction mix containing SYBR Green qPCR 1 x Master Mix with ROX (KAPA SYBR FAST qPCR Kit, KAPA BIOSYSTEMS, USA) and 0.3 µM of each primer.

Primer sequences used for the Real-Time qPCR were mRNA specific with following sequencing: U6 Forward GCTTCGGCACGACATACTAAAAAT; Reverse CGTTCAGAATTTGGCTGTAC; MGP Forward TGGAGCTCTGACCCAGCAAG. Analysis was performed on Applied Biosystem® 7500 Real-Time qPCR instrument (USA). The amount of mRNA of each gene of interest was normalized according to the amount of mRNA encoding U6 as an internal control. Gene expression levels were calculated using 2−ΔΔCt method [23]. This method represents a relative gene expression levels. Relative quantification relates the PCR signal of the target transcript in the samples from a study group to that of a calibrator group, where expression is equal to 1. For the needs of relative gene expression analysis in this study, nine volunteers represented a calibrator group of healthy individuals, with no documented present or history of CVD disease. The calibrator group was different from the study group (n=87). Individual MGP mRNA levels of each participant in the survey (n=87) were calculated regarding the calibrator.
Statistical analysis

Data were presented as median with range (min–max), or number (n) and percentage (%), as appropriate. Data distribution (normal/non-normal) was tested using Kolmogorov-Smirnov Z test.

Standard statistical methods – descriptive statistics, Mann-Whitney and Kruskal-Wallis analysis for non-Gaussian distributed data, t-test for normally distributed interval data, and one-way analysis of variance (ANOVA) including Bonferroni correction were used. The categorical variables were analyzed using Chi-square test. Relationship between continuous variables was evaluated by Spearman’s correlation analysis. All statistical analyses were two tailed. p-Values <0.05 were considered statistically significant. Data analysis was performed on GraphPad Prism v. 8.3., USA and SPSS v. 23, USA.

Results

Baseline characteristics of the studied patients

Baseline characteristics of the participants in the study are presented in Table 1 (Supplementary Material). The mean age of all participants was 62.12 ± 1.28. The females dominate in our study. They were 65.5% of all patients. Obesity (BMI ≥ 30 kg/m^2) was observed in 32.8% of the entire group with prevalence in CVD group (43.8%). Smokers dominated in the control group (54.1%) vs. (24.2%) in the CVD group (p<0.05). CACS was higher in the CVD group vs. controls (p=0.0001). More than 80% of the studied patients were with arterial hypertension (AH) with median duration 10 years (IQR 3.5–15 years). Hyperlipidemia was present in more than 70% of the studied patients. The levels of non-protein nitrogen-containing compounds (urea, creatinine, uric acid) tend to increase in the CVD group compared to the controls. Significant elevation was found in creatinine (p=0.0002) and uric acid (p=0.013) levels for CVD patients vs. controls. Of the tested lipid parameters, significant changes were found for TC (p=0.015), LDL-C (p=0.0003), and LDL-C/HDL-C ratio (p=0.007). Changes with borderline significance were found for TC/HDL-C ratio. Circulating ucMGP tend to be higher in the CVD patients by 83.3% (p=0.081).

Expression of MGP in PBMC according to CVD severity

CVD subgroup included patients with atrial fibrillation (AF) and those with heart failure with preserved ejection fraction (HF). Due to the small number of patients within AF and HF group and the absence of significant differences between them and the controls in regards to MGP expression, we combined AF and HF patients in one CVD subgroup. When analyzed, the differences in the MGP gene expression between controls and CVD-group were non-significant (p=0.402). The expression levels in the CVD group were slightly lower (by 30.0%) when compared to the controls (0.98; 0.18–17.73 RU mRNA vs. 1.40; 0.12–16.83 RU mRNA) (Figure 1). We observed significantly higher (by 29.4%) ucMGP expression in the patients with CACS>0 AU as compared to those with CACS=0 (1.043; 0.12–17.73 RU mRNA vs. 0.736; 0.004–3.51 RU mRNA, p=0.032) (Figure 2A). When the CACS values were subdivided into tertiles, a gradual increase in ucMGP expression with the elevation of CACS was found reaching significantly highest value for CACS=100 AU (0.736; 0.004–3.514 RU mRNA vs. 1.106; 0.183–17.73 RU mRNA; p=0.037) (Figure 2B).

Expression of MGP in PBMC according to etiologic risk factors for CVD

MGP gene showed differential expression according to presence of conventional CVD factors (Figure 3). Although non-significant, a trend for a lower MGP expression in PBMC of the individuals in the study group (n=87) was observed with age (by 13.0%, p=0.65), in female patients (by 16.0%, p=0.57), with BMI (by 8.2%, p=0.82), WC (by 30.5%, p=0.23), with the presence of AH (by 44.5%, p=0.12) and duration of AH (37.6%, p=0.25) and with presence of HL (by 25.0%, p=0.40). We found most dramatical decrease in MGP expression with the presence of AH, abdominal obesity, and the presence of HL, risk factors considered more prone to CVD development and progression. Due to the small number of patients in these groups, the differences were non-significant. We did not find differences in MGP expression according to smoking status.
Expression of MGP in PBMC according to metabolic risk factors for CVD

Figure 4 represents MGP mRNA levels in PBMC in the study group (n=87) according metabolic risk factors for CVD. MGP expression was significantly lower in patients representing high TC and LDL-C levels by 51.0% (p=0.02) and 50.6% (p=0.04), respectively. Similarly, a trend for lower MGP expression according to the levels of the tested metabolic risk factors representing higher CVD risk was observed for all other subgroups, except HDL-C. Diminishment of MGP expression we observed for TC/HDL-C (by 24.4%, p=0.39), for LDL-C/HDL-C (by 17.0%, p=0.57), and for UA (by 16.7%, p=0.56). Surprisingly, ucMGP expression was non-significantly higher (by 22.7%) in patients with HDL-C levels above cut-off median value of 1.36 mmol/L (Figure 4).

Expression of MGP in PBMC according to circulating ucMGP plasma levels

Higher ucMGP levels in plasma were negatively associated with MGP expression levels in PBMCs. MGP mRNA levels were lower in the subgroups with plasma concentrations over the respective cut-off median values of circulating ucMGP in the total group (by 19.7%, p=0.47) and both the control (33.5%, p=0.34) and CVD group (25.7%, p=0.50) (Figure 5).
Correlation analyses

Although MGP expression analysis did not reveal a significant difference between smokers and nonsmokers among our cohort, the correlation analysis showed association of MGP expression in PBMC and the number of cigarettes per day for the patients with coronary calcium ($\rho=0.554$, $p<0.01$) and in the CVD group ($\rho=0.731$, $p<0.01$). AH duration correlated negatively with MGP mRNA levels ($\rho=-0.312$, $p<0.053$) in the group without coronary calcium deposits. Linear regression analysis did not find significant associations between circulating ucMGP levels and MGP expression in PBMC.

Discussion

In the current study, we aimed to assess the expression of MGP in PBMC in adult patients with different CVD pathologies and its association with the presence of CACS and with conventional CVD risk factors. MGP gene expression tends to decrease with deepening the CVD pathology and with some of the CVD risk factors. MGP gene expression in PBMC was down regulated in obese, hypertensive, and hyperlipidemic patients with high total and LDL-cholesterol.

PBMC are an useful and non-invasive tool to study gene expression profile. Unfortunately, there is very limited number of reports on their usage as a tool for testing biomarkers related to different CVD pathologies, and even less are the studies related to MGP expression in PBMC in CVD patients. Studies on PBMC expression profiles in rat models of induced hypertensive heart disease revealed dramatical changes affecting signaling cascades for cell cycle regulation, tissue repair, inflammation, and redox balance maintenance [24]. A recent study reported lower MGP mRNA levels in diseased aortic valve interstitial cells vs. healthy valve cells [25]. Similarly, PBMC gene expression has been established to be altered in patients with pre-heart failure [26], heart failure [27], chronic heart failure [28, 29], congestive heart failure [30], and advanced congestive heart failure [31]. All these findings support the hypothesis that PBMC reflect the CVD pathology and may be used as a potential tool for biomarkers testing in CVD. Our data reveal a tendency for a decreased MGP expression in PBMC, though not very convincingly, support this hypothesis.
As the changes in MGP gene expression related to the levels of the circulating MGP, we tested the relationship between these two parameters. We did not find a significant relationship between circulating MGP levels and MGP expression in PBMC in the total study group (n=87) and for both controls and CVD group. Non-significant difference in MGP expression was established according to CVD pathology. We observed significantly higher MGP expression in the patients with CACS>0 AU as compared to those with CACS=0 (Figure 2A). A gradual increase in MGP expression with the elevation of CACS was found reaching significantly highest value for CACS>100 AU (Figure 2B).

We found no data in the literature regarding the relationship between MGP expression in PBMC and circulating MGP. Furthermore the data in the literature regarding the associations of circulating MGP levels with CVD pathology and vascular calcification are highly controversial. Total serum MGP has been found to be higher in patients with ischemic heart disease [32], in postmenopausal women with carotid calcification [33], in patients with severe atherosclerosis and arterial calcification [34]. Other studies indicated that circulating MGP levels were not correlated with the presence of CAC in healthy women [8]. Lower MGP expression in PBMC was established for the patients with AH as compared to normotensive individuals. In the present study, AH duration correlated negatively with MGP mRNA levels (r=-0.312, p<0.053) in the patients without coronary calcium. It can be hypothesized that MGP expression in PBMC is down regulated by deepening the risk factors for CVD. Although the expression of MGP in PBMC very slightly contributes to the circulating MGP levels, we could speculate that with the progression of CVD the levels of the newly synthesized MGP will diminish resulting in a decreased substrate for the formation of the active p-cMGP form.

Obesity is recognized as an independent risk factor for CVD and one of the main causes for increased risk of dyslipidemia, insulin resistance, hypertension, and atherosclerosis [35]. We found a decrease of MGP expression with obesity indexes more pronounced in patients with abdominal obesity (by 30.5%, p=0.40) with deepening the hyperlipidemia (elevated TC and LDL-C). In our cohort we established significantly lower MGP expression in the subgroups with high total and LDL cholesterol by 51.0% (p=0.02) and 50.6% (p=0.04), respectively. These findings, together with the remarkable trend for lower MGP expression according to all of the studied risk factors in the groups with higher CVD risk, support the hypothesis that MGP expression in PBMC probably reflects CVD pathology and is related to dysregulation of lipid metabolism. Although the associations between MGP expression, circulating MGP and obesity parameters are not fully understood, there are data revealing negative correlation of serum MGP with LDL-C and LDL-C/HDL-C ratio in patients with ischemic heart disease [32] and positive relation with HDL-C in subjects with coronary syndrome [38]. In contrast, a reverse relationship between serum MGP and HDL-C levels and a positive association with TC/HDL-C ratio and TC was found in healthy subjects [10]. Our findings, although not very convincing, also demonstrate such relationships between lipid parameters and MGP expression in PBMC in CVD individuals. Further studies would reveal involvement of MGP in body mass regulation and contribution of PBMC in this process.

Additional insight for the relationship between MGP expression in PBMC and CVD risk factors provides the moderate correlation between MGP expression in PBMC and the number of cigarettes per day in CVD patients with coronary calcium. Although there is no evidence in the literature how smoking status is related to MGP expression, there are data for associations of smoking and circulating MGP levels that are indirectly consistent with our results [10].

To our knowledge, this is the first study attempting to assess the relationships between the expression of MGP in PBMC CVD pathology, vascular calcification evaluated by Agatston CACS and conventional CVD risk factors. An interdisciplinary approach was used for the analysis of clinical parameters in CVD patients in relation to biochemical and molecular biology markers.

One of the limitations of the study is that MGP gene expression in PBMC was studied on transcriptional level only. Studying intracellular total MGP protein content and its posttranslational isoforms would provide more...
complete insight for their relation to CVD pathology and risk factors. Furthermore, in case of higher number of participants in the cohort, a better statistical significance for more parameters would be expected.

Conclusions

The current study support the hypothesis that MGP expression in PBMC probably reflects CVD pathology and is related to lipid metabolism dysregulation. MGP gene expression in PBMC tends to decrease with deepening the CVD pathology and is down regulated in obese, hypertensive, and hyperlipidemic patients with high total and LDL-cholesterol. Further studies are needed to clarify the applicability of MGP gene expression in PBMC as a potential biomarker for the CVD assessment.

Research funding: This study was supported by “Science” fund, Grant No 17002, Medical University “Prof. Dr. Paraskev Stoyanov”-Varna, Bulgaria; “Medical University - Varna”.

Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: Authors state no conflict of interest.

Informed consent: All participants provided written informed consent prior to participate in the study.

Ethical approval: The research related to human use has complied with all the relevant national regulations, institutional policies, and in accordance with the tenets of the Helsinki Declaration, and has been approved by the author’s Institutional Review Board – Ethical Committee at the Medical University of Varna (Protocol No 75/07.06.2018).

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**Supplementary Material:** The online version of this article offers supplementary material (https://doi.org/10.1515/tjb-2021-0167).