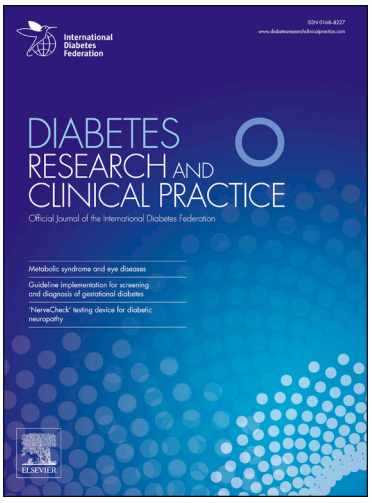

Association of matrix γ-carboxyglutamic acid protein levels with insulin resistance and Lp(a) in diabetes: A cross-sectional study

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ASSOCIATION OF MATRIX γ-CARBOXYGLUTAMIC ACID PROTEIN LEVELS WITH INSULIN RESISTANCE AND Lp(a) IN DIABETES : A CROSS-SECTIONAL STUDY

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ABSTRACT

Aims:The risk of cardiovascular disease (CVD) and mortality is increased in patients with chronic kidney disease (CKD), with a background role of vascular calcification in the development of CVD also reported. Studies have demonstrated that high lipoprotein(a) (Lp(a)) levels accelerate the development of atherosclerolsis and are potentially involved in the vascular calcification.Matrix Gla Protein (MGP) seems to play an important role in vascular calcification. The aim of the study was to examine the potential association of MGP concentrations with Lp(a) and insulin resistance.
Methods:The study involved 100patients divided in four groups: 25 with both CKD stage 4 and Type2 Diabetes (DM) (Group-A), 25 with CKD4 without DM (Group-B), 25 non uremic patients with DM (Group-C) and 25 healthy subjects (Group-D). Serum glucose, Lp(a), MGP, plasma HBA1c and insulin were measured in all patients. Insulin resistance was estimated by the homeostasis model assessment equation(HOMA-IR).
Results:A significant positive linear association between MGP and Lp(a) levels (r=0.272,*p*=0.006) was noted, as well as between MGP and HOMA-IR levels (r=0.308,*p*=0.002). However, no significant linear association between Lp(a) and HOMA-IR levels was recorded. A similar positive association between MGP and insulin levels (r=0.204,*p*=0.042) was also found.
Conclusion:This study concluded that diabetes coexisting with renal disease leads to extreme vascular calcification expressed by elevated MGP levels, resulting in higher frequency of cardiovascular disease in comparison to CKD patients without diabetes. The detected Lp(a) and MGP association in CKD4 patients may also represent the key to the complicated mechanism of their coexisting accelerated atherosclerosis and vascular calcification.

Keywords: Matrix γ-carboxyglutamic acid protein (MGP); insulin resistance; lipoprotein (a); diabetes mellitus type 2; chronic kidney disease.

1. Introduction

It is commonly accepted that the risk of cardiovascular disease and mortality is increased in patients with chronic kidney disease (CKD) [1,2]. Cardiovascular disease (CVD) in CKD is associated with arteriosclerosis and arterial stiffness [2,3]. Moreover, studies have pointed out the crucial role of vascular calcification in the development of CVD especially in certain patient groups, such as those with CKD [4,5]. It should be noted that cardiovascular calcifications in patients with CKD are more prevalent (up to fivefold more excessive vascular calcification), progressive and severe compared to non-CKD population or age-matched individuals with coronary artery disease [6,7].

Vascular calcification is developed when calcium/calcium phosphate complexes deposition occurs in the intima and/or media layers of the vessel and/or valvular tissue once they have become mineralized [8]. Pathogenesis of vascular calcification is believed to be a multifactorial and multi-step process [9,10]. Numerous factors associated with vascular calcification have been identified including duration of hemodialysis, hypercalcemia, hyperphoshphatemia, increased calcium x phosphorus (Ca x P) product, hyperparathyroidism, diabetes mellitus, dyslipidemia, high levels of lipoproteins, urea, homocysteine, parathormone, inflammation, oxidative stress and patient's age [11,].

Pathophysiology and etiopathogenesis of vascular calcification and atherosclerosis, in patients with chronic kidney disease remains unclear. It has been suggested that a disturbed equilibrium between calcification promoters and inhibitors may possibly lead to vascular wall calcification [2]. Vascular calcification process may also include the differentiation of vascular smooth muscle cells (SMCs) into osteoblast-like cells possibly due to uremic toxins which may accelerate core binding factor a-1 (Cbfa1) expression [13]. Other factors involved in the development of vascular calcification are the main calcification inhibitory molecules, such as fetuin-A, osteoprotegerin (OPG), and the Matrix Gla Protein (MGP) [10,14].

MGP, a low molecular weight protein originally isolated from bone, is a vitamin K-dependent extracellular matrix protein, known to be synthesized by chondrocytes in the cartilage and by vascular smooth cells in the arterial vessel wall [15,16]. MGP is involved in modulating vascular calcium metabolism. It belongs to a protein family that contains γ-carboxyglutamic acid residues (Gla) which play a key role in its calcium binding capacity [17], while undercarboxylated MGP levels were inversely associated with the calcification process in patients undergoing hemodialysis [18].

MGP was the first in vivo inhibitor of vascular calcification discovered, expressed in abundance in atherosclerotic plaques, although it is present in normal artery wall as well. It has been demonstrated that MGP acts as an inhibitor to soft tissue calcification [4,19] showing also an inverse association with the severity of vascular calcification [20] however, high levels of MGP were detected not only in normal blood vessels, but also in calcified human atherosclerotic plaques [21,22]. According to previous studies [23,24], uncarboxylated MGP (probably) due to deficiency of vitamin K (especially menaquinone K2), as well MGP gene polymorphisms [25,26] are associated with the development or progression of calcification and may be related to the pathogenesis of cardiovascular events.

Thus, CKD and dyslipidemia are two significant risk factors for cardiovascular disease and atherosclerotic events. Further, metabolic syndrome and diabetes

mellitus are recognized as two major risk factors for the development not only of cardiovascular disease, but also of CKD [27,28].

Lipoprotein(a) (Lp(a)), a low density lipoprotein, is associated with cardiovascular events and may also represent a link between thrombosis and atherosclerotic disease process [29,30]. Studies have also demonstrated that high Lp(a) levels, accelerate the development of atherosclerolsis and are potentially involved in the vascular calcification [31,32]. In addition, Lp(a) has been identified closely in the calcified lessions of atherosclerotic areas and a potential effect of Lp(a) on human vascular smooth muscle cells has been also documented indicating that Lp(a) may be involved in vascular calcification [32,33].

Given the fact that MGP, synthesized by chondrocytes and vascular smooth muscle cells, plays a key role in the inhibition of tissue calcification we tried to examine the potential associations of MGP concentrations with Lp(a) levels and insulin resistance in patients with CKD and/or DM.

2. Material and methods

2.1 Study population

This study involved 100 participants, recruited through convenience sampling attending the outpatient clinics of the involved hospitals. Design was cross-sectional including cases and controls. Subjects were divided in four groups: Group A consisted of 25 patients with stage 4 chronic kidney disease (CKD4) and diagnosed type 2 Diabetes Mellitus (DM), Group B consisted of 25 patients with stage 4 chronic kidney disease without diabetes, Group C included 25 patients with DM without impaired renal function, and Group D consisted of 25 patients, without chronic kidney disease, diabetes or any other major chronic conditions. The subjects of this group were considered as healthy subjects (HS).

All patients included in the study were adults. Inclusion criterion for uremic patients was GFR 15-29 ml/min/1.73m^2. All patients without diabetes had normal fasting glucose levels, normal HBA1c levels and normal glucose tolerance. The study was approved by the Local Ethics Committee of the participating hospitals and performed in accordance with the Declaration of Helsinki. Written informed consent was obtained by all individuals. Data were stored in a secured server, all tests contributing to the study database were appropriately anonymised and only administrative staff had access to the identifiable files.

2.2 Laboratory measurements

Medical history and anthropometric parameters were obtained from all individuals. In addition, fasting venous blood samples were collected from all participants for determining biochemical parameters. Serum glucose, electrolytes, cholesterol and plasma HBA1c were measured on an architect (ABBOTT) 16200 analyzer.

Serum samples were stored at -80C until process. Lp(a) was measured by immunonephelometry using the Abbott Architect ci 16200 analyzer (Abbott Park, IL,

USA). Immunonephelometry is also mentioned as a way of Lp(a) measurement in previous studies [34,35], while being based on previously reported data, Lp(a) concentrations >30 mg/dL were indicative of high risk patients [35, 36].

Plasma insulin was estimated by Microparticle enzyme kits (ABBOTT IMx Insulin assay) on an IMx ABBOTT analyzer. GFR was calculated with the MDRD equation [37] and insulin resistance was estimated by the homeostasis model assessment equation (HOMA-IR) as described by Matthews et al. [38]. The levels of hs-CRP in the serum samples were measured with high-sensitivity methods using nephelometry (BN II Nephelometer-Siemens). MGP levels were measured with ELISA method (Biomedica Gruppe, Wien, Austria).

2.3 Statistical analysis

Data are shown as mean ± SD (standard deviation) or as numbers (n). All the variables were tested for normal distribution using the Kolmogorov-Smirnov test. Analysis of variables (ANOVA) was used to compare differences in the studied parameters among the four groups. P-values < 0.05 (two-tailed significance) were considered statistically significant. When a P-value < 0.05 was found in ANOVA, the post hoc LSD was used to look for differences between the study groups. Categorical variables were compared by the chi-square test. In order to trace possible linear associations between the variables the Pearson correlation coefficient was measured. Analysis of data was performed using SPSS 15.0 (Lead Technology Inc).

3. Results

The four groups did not differ significantly in terms of gender, age, waist circumference and history of arterial hypertension. However, a statistically significant difference in terms of smoking habits (p=0.02), history of heart disease (p<0.001) and ischemic stroke (p=0.01) was indicated for group A. Hematocrit, serum levels of MGP, high sensitivity CRP (hsCRP), total cholesterol, HDL, LDL, TGs Lp(a), Insulin, electrolytes (Ca, P), plasma levels of HbA1c and insulin resistance estimated with HOMA-IR were determined for the four groups and they were compared using one-way ANOVA methodology to detect a statistically significant difference of the means between the groups. Demographic and clinical characteristics of the study subjects are shown in Table.

Table

Main demographic and clinical characteristics of the study population (total number of participants=100)

	Group A (CKD4+DM)	**Group B (CKD4)**	**Group C (DM)**	**Group D (HS)**	***p*-value**
Gender (men/women)	12/13	14/11	11/14	11/14	NS
Age (years)	67.9±7.4	65.7±12.3	67.7±6.5	65.3±8.1	NS
Waist circumference (cm)	100.7±10.2	94.2±10.4	97.9±11.2	98.3±9	NS
Heart Disease (Y/N)	12/13[a,b,c]	6/19[a]	2/23	0/25	<0.001
Ischemic stroke (Y/N)	8/17[a,c]	1/24	4/21	0/24	0.01
Smoking (Y/N)	9/16[b]	11/14[b]	2/23	6/19	0.02
Dyslipidemia (Y/N)	14/11	9/16	18/7	15/10	NS
Hypertension (Y/N)	25/0	23/2	21/4	23/2	NS
Diabetes mellitus (Y/N)	25/0	0/25	25/0	0/25	
GFR (ml/min/1.73m^2)	17.1±1.51[a,b]	17.3±1.52[a,b]	76.8±10.06	76.4±10.84	<0.0001

Insulin (µUI/ml)	22.04±15.0[a,b]	18.16±12.6	12.32±6.49	12.44±7.65	0.005
HbA1c (%)	7.3±1.2[a,c]	5.6±0.3[b]	7.8±1.3[a]	5.8±0.3	<0.0001
HOMA	6.92±6.0[a,b,c]	4.12±3.02	4.73±2.96	2.94±1.86	0.003
Lp(a) (mg/dl)	45.15±50.0[a,b]	34.98±25.3[a]	21.04±23.6	10.12±7.22	0.001
MGP (nmol/l)	6.22±2.20[c]	4.84±1.5	5.66±1.12	5.58±0.92	0.01
hsCRP (mg/l)	21.56±31.4[a.b]	13.5±24.0	7.55±13.4	3.54±4.03	0.01
Ca (mg/dl)	7.64±0.91[a,b,c]	8.60±0.72[a,b,d]	9.38±0.50	9.31±0.40	<0.0001
P (mg/dl)	5.28±1.17[a,b]	4.92±1.16[a,b]	3.35±0.39	3.30±0.54	<0.0001
CHOL (mg/dl)	163.6±42.8[a.b]	173.6±46.7[a,b]	201.8±41.6	201.7±43.3	0.003
HDL (mg/dl)	42.93±12.6[b]	41.24±11.5[a,b]	49.6±11.5	48.28±10.5	0.03
LDL (mg/dl)	95.7±37.2[a,b]	107.9±39.5	130.3±33.1	129.5±36.9	0.002
TGs (mg/dl)	125.0±52.9	122.6±51.2	109.6±43.7	119.6±69.0	NS

CKD4, chronic kidney disease stage 4; DM, Diabetes Mellitus; HS healthy subjects

Heart disease is medical history of myocardial infarction, angina, coronary intervention procedures, heart failure.

[a]statistically significant compared to HS, [b]statistically significant compared to DM

[c]statistically significant compared to CKD4, [d]statistically significant compared to CKD4+DM

Differences are statistically significant at the 0.05 level

Insulin resistance was higher in CKD4+DM (group A) in comparison to CKD4 (group B), DM (group C) and healthy subjects (group D). In addition, HbA1c levels were significantly higher in patients of group A compared to group B and group D.

MGP serum levels were significantly higher in CKD4+DM patients compared to uremic patients without diabetes (CKD4). However, no significant difference of MGP serum levels among the other groups was indicated. Lp(a) was found to be significantly higher in patients with renal disease stage 4, both with or without diabetes mellitus, compared to healthy subjects. Moreover, Lp(a) was significantly higher in CKD4+DM patients compared to patients with diabetes without renal failure.

Total population analyses were performed in order to identify potential associations among the parameters. In fact, there was a weak, although highly significant, positive linear association between MGP and Lp(a) levels (r=0.272, *p*=0.006), as well as similarly strong association between MGP and HOMA IR levels (r=0.308, *p*=0.002). A similarly positive association between MGP and insulin levels (r=0.204, *p*=0.042), was also found. However, no significant linear association between Lp(a) and both HOMA IR and insulin levels was indicated (r=0.122, *p*=0.097 and r=0.173, *p*=0.085, respectively). Given these results we performed further subgroup analyses for each category separately and the only association observed was between MGP and LP(a) levels only in group A (r=0.455, *p*=0.022).

4. Discussion

This study outlines that MGP and Lp(a) levels, which are indicative of vascular calcification and atherogenetic process, play an important role in patients with CKD regardless of a diabetes diagnosis. The majority of patients with chronic kidney disease (CKD) have excessive vascular calcification, which is often considered a strong prognostic marker of cardiovascular disease and mortality.

Our study showed that patients with stage IV CKD and DM have significantly higher serum levels of MGP compared to uremic patients without diabetes. However, no significant difference in MGP levels was found between the latter and healthy subjects. This finding demonstrates that diabetes mellitus, coexisting with renal disease, leads to extreme vascular calcification, expressed by elevated MGP levels, resulting in the higher frequency of cardiovascular disease in these patients compared to patients with CKD and without DM.

These results concur with a previous study, which found that serum levels of MGP were higher in patients with a high number of risk factors for coronary artery disease as well as in patients with high overall Framingham coronary heart disease risk score [39]. Furthermore, Braam et al. found that levels of circulating MGP in patients with type 1 Diabetes Mellitus (which is a risk factor for atherosclerosis) were elevated [40]. Our findings concerning the group B of patients (CKD patients without diabetes) are in agreement with the results of Jono et al, which detected a reverse association between the levels of serum MGP and coronary artery calcification [9].

Possible explanations of the above findings is that MGP may be induced in response to calcification as a local negative feedback mechanism that increases MGP levels in calcified human plaque. Thus, a part of MGP that is water-soluble is released in circulation and may account for the elevated levels of serum MGP

observed in atherosclerotic patients. Another potential mechanism is inadequate levels or activity of mineralization inhibitor such as MGP in CKD patients [41].

Additionally, Lp(a) levels were significantly higher in patients with CKD (with or without diabetes) compared to diabetic patients without CKD or healthy participants. It should be noted that patients in both categories (namely, CKD+DM and CKD) demonstrated particularly elevated levels of Lp(a), indicative of being high risk patients [35,36] , while the coexistence of both CKD and DM was accompanied with even higher levels of Lp(a), emphasizing the notably increased cardiovascular risk in those patients.

Moreover, an independent and statistically significant positive linear association was reported between MGP and Lp(a), as well as MGP and HOMA-IR in the total sample population. This suggests an in vivo association of the vascular calcification process with atherogenesis and with the insulin resistance pathophysiological spectrum. However, in subgroup analyses an association between MGP and Lp(a) was observed only in patients with CKD and DM, supporting the findings that renal disease and DM comorbidity is associated with an even higher cardiovacsular risk.

Lp(a) levels appeared to be significantly increased in patients with CKD, possibly assosiated with genetic as well as other factors related to CKD apart from GFR [42]. On the other hand, Lp(a) concentrations were not significantly correlated with insulin sensitivity (HOMA-IR) which is consistent with existing literature [43-44].

The association between MGP and Lp(a) was consistent with the results of a previous study performed in transgenetic rabbits in which Lp(a) levels were associated with increased alkaline phosphatase activity and calcium accumulation [32]. Moreover, a positive association was also observed in patients with cardiovascular disease [45]. A possible explanation could be that Lp(a), and the subsequently induced vascular calcification promote the MGP expression by smooth muscle cells possibly reflecting a transformation of vascular smooth muscle cells to osteoblast-like cells especially in patients with CKD [46].

In addition, the recorded significant positive linear association between MGP and HOMA-IR reflects the increased vascular calcification process observed in patients with diabetes. The pathogenesis of vascular calcification in diabetes is not completely understood, though high glucose and other potential factors may play an important role by transforming VSMCs into osteoblast-like cells [47]. Our findings are consistent with Parker's et al who found significantly higher levels of uncarboxylated MGP (the precursor of MGP) associated with mitral annular calcification and aortic stenosis in patients with diabetes compared to those without diabetes [48]. This finding supports the hypothesis that MGP plays a key role in the increased vascular calcification process observed in patients with diabetes. Further understanding of the mechanism by which diabetes induces this complication is needed to design effective therapeutic strategies to intervene with this process [46].

Finally, according to our findings a parallelism between hsCRP and Lp(a) is noted. Lp(a) is associated with atherosclerosis, in which inflammation process has also been implicated in the pathogenesis of coronary artery disease. hs-CRP is a sensitive index of inflammation, however, there is not sufficient scientific bibliographical support to indicate a direct association between the latter (namely hsCRP) and Lp(a) [49-50]. As a result, due to the limitation of our study and the

potential confounding factors between the two parameters, more research is needed before any conclusion can be drawn.

Regarding the limitations of the present study, the relatively small sample size may diminish the statistical power and certain secondary observations may not have reached statistical significance. Due to lack of resources in recruitment, patient matching by predictors of vascular calcification could not be done. Moreover, the absence of vascular atherosclerosis estimation and the cross-sectional design does not allow the substantiation of an etiological or pathophysiological hypothesis.

However, to the best of our knowledge, this is the first study that examined the MGP levels simultaneously in these four categories and tried to investigate possible associations with insulin resistance and Lp(a) levels.

5. Conclusion

In conclusion, we have demonstrated that in patients with CKD, the Lp(a) were significantly elevated. This is an important finding as the Lp(a) and MGP levels association may represent the key to a complicated mechanism of the coexisting accelerated atherosclerosis and vascular calcification development in those patients. Excessive vascular calcification occurs when diabetes mellitus coexists in CKD patients, resulting in higher risk of major cardiovascular events and mortality. MGP plays an important, protective, role against this process by acting as a vitamin k-dependent inhibitor for vascular calcification. However, the mechanism behind the vascular effect of MGP in CKD patients without diabetes mellitus would require further research exploration.

Authors Contributions

Stavros Antonopoulos, Maria Mylonopoulou, Angeliki M. Angelidi and Nicholas Tentolouris planned and conducted the current study. Stavros Antonopoulos, Maria Mylonopoulou and Angeliki M. Angelidi wrote the manuscript. Antonis A. Kousoulis, Nicholas Tentolouris edited the manuscript. Stavros Antonopoulos, Maria Mylonopoulou and Angeliki M. Angelidi contributed equally to this work and share the first authorship. All the authors read and approved the final paper.

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Highlights

- Diabetes coexisting with chronic kidney disease is associated with elevated MGP levels
- A positive association between MGP and both Lp(a) and HOMA IR levels was found
- Potential association of Lp(a) and MGP with atherosclerosis & vascular calcification