ORIGINAL ARTICLE

VASCULAR CALCIFICATION AND GROWTH ARREST SPECIFIC PROTEIN 6 LEVELS IN CHRONIC RENAL DISEASE

CALCIFICACIÓN VASCULAR Y NIVELES DE LA PROTEÍNA ESPECÍFICA DEL GEN 6 DE LA DETENCIÓN DEL CRECIMIENTO EN LA ENFERMEDAD RENAL CRÓNICA

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ABSTRACT

Introduction: Cardiovascular disease is the main cause of mortality and morbidity in chronic renal failure. It's known that vascular calcification (VC) and carotid intima media thickness (CIMT) are strongly associated with cardiovascular diseases. Growth arrest specific protein 6 (Gas6) is a vitamin K-dependent protein and regulates various processes such as proliferation, cell survival, migration and inflammation. Gas6 is known to protect endothelial cells and vascular smooth muscle cells against apoptosis by inhibiting Bcl-2 induced Caspase 3 activation. The relationship between Gas6 and cardiovascular diseases has been demonstrated in many mouse models and cell cultures. However, there are conflicting reports whether Gas6 levels are increasing or decreasing in human studies of diabetic and/or chronic renal failure. In present study the aim was to examine plasma Gas6 levels and its relation with CIMT and coronary artery calcification score (CACS) in chronic kidney disease (CKD) patients. **Methods:** Total of 137 patients of which 32 chronic hemodialysis and 105 predialysis patients as well as 73 healthy controls were enrolled in the study. Human Gas6 levels in serum samples were studied by ELISA method. CIMT was measured by ultrasonography. CACS was measured by multislice computed tomography. **Results:** The mean age was 54.37±16.61 years in dialysis group, 55.20±14.80 years in predialysis group and 53.26±9.04 years in control group. Serum creatinine was 0.78±0.16 mg/dl in the control group and 1.96±1.64 mg/dl in the predialysis and 5.94±1.55 mg/dl in the dialysis group. 24 hours urine protein levels were significally higher in the dialysis group than the predialysis and the control group. CIMT values were similar in predialysis and dialysis groups. These values were significantly higher than control group. Although CACS was higher in dialysis group than predialysis and control group, the results were not statistically

significant since the distribution range was very wide. Gas6 was 98.84±53.32 ng/mL in the control group and statistically higher than dialysis (63.85±38.92 ng/mL) and predialysis groups (54.96±38.49 ng/mL) (p=0.001). Gas6 levels were lower in diabetic patients than non-diabetics (53.69±35.26 ng/mL, 69.26±47.50 ng/mL, p=0.023, respectively). Negative correlation was detected between Gas6 and age, BMI, CACS, carotid IMT and proteinuria. In the logistic regression analysis, Gas6 remained significantly associated with BMI, CIMT and proteinuria. **Conclusion:** In our study, a negative correlation of Gas6 with BMI, CACS, CIMT and proteinuria and lower Gas6 levels in diabetic patients support that decreased Gas6 levels in chronic renal failure may have a role in vascular calcification through altered glucose tolerance, chronic inflammation, endothelial dysfunction and increased apoptosis. Our study has an importance because it is the first study showing a relation between Gas6 and proteinuria, CACS and carotid IMT in patients with chronic renal failure.

KEYWORDS: carotid intima media thickness; chronic kidney disease; coronary artery calcification score; growth arrest specific protein 6; vascular calcification

RESUMEN

Introducción: La enfermedad cardiovascular es la principal causa de mortalidad y morbilidad en la insuficiencia renal crónica. Se sabe que la calcificación vascular (CV) y el grosor de la íntima-media de la carótida (CIMT, por sus siglas en inglés) están vinculados de forma muy estrecha con enfermedades cardiovasculares. La proteína específica del gen 6 de la detención de crecimiento (Gas6) es una proteína dependiente de la vitamina K y regula diversos procesos, como la proliferación, la supervivencia celular, la migración y la inflamación. La proteína Gas6 es conocida por proteger a las células endoteliales y las células musculares lisas vasculares contra la apoptosis mediante la inhibición de la activación de la caspasa-3 inducida por la proteína Bcl-2. Se ha demostrado la relación entre la Gas6 y las enfermedades cardiovasculares en muchos modelos de ratones y cultivos celulares. Sin embargo, existen informes contradictorios acerca de si los niveles de Gas6 aumentan o disminuyen en estudios de humanos con insuficiencia renal crónica y/o diabetes. En este estudio, el objetivo fue examinar los niveles plasmáticos de Gas6 y su relación con el CIMT y la puntuación de calcificación de las arterias coronarias (CACS, por sus siglas en inglés) en pacientes con enfermedad renal crónica (ERC). **Material y métodos:** Un total de 137 pacientes fueron incluidos en el estudio, de los cuales 32 estaban en hemodiálisis crónica, 105 en prediálisis, y 73 pacientes representaban controles sanos. Se estudiaron los niveles de Gas6 en muestras de suero mediante el método ELISA. El CIMT se midió por medio de ecografía. La CACS se midió mediante tomografía computarizada multicorte. **Resultados:** La edad media fue de 54,37 ± 16,61 años en el grupo de diálisis; 55,20 ± 14,80 años en el grupo de prediálisis, y 53,26 ± 9,04 años en el grupo de control. La creatinina sérica fue de 0,78 ± 0,16 mg/dl en el grupo de control; 1,96 ± 1,64 mg/dl en el de prediálisis, y 5,94 ± 1,55 mg/dl en el de diálisis. Las concentraciones de proteína en orina de 24 horas fueron significativamente más altas en el grupo de diálisis que en los de prediálisis y control. Los valores del CIMT fueron similares en los grupos de prediálisis y de diálisis. Estos valores fueron considerablemente más altos que en el grupo de control. Aunque la CACS fue más alta en el grupo de diálisis que en los otros dos, los resultados no fueron estadísticamente significativos, ya que el rango de distribución fue muy amplio. La proteína Gas6 fue de 98,84 ± 53,32 ng/ml en el grupo de control y estadísticamente más alta que en los grupos de diálisis (63,85 ± 38,92 ng/ml) y de prediálisis (54,96 ± 38,49 ng/ml) (p = 0,001). Los niveles de Gas6 fueron más bajos en los pacientes diabéticos que en los no diabéticos (53,69 ± 35,26 ng/ml; 69,26 ± 47,50 ng/ml, [p = 0,023], respectivamente). Se detectó una correlación negativa entre la proteína Gas6 y la edad, el IMC, la CACS, el CIMT y la proteinuria. En el análisis de regresión logística, la Gas6 se mantuvo

estrechamente relacionada con el IMC, el CIMT y la proteinuria. **Conclusión:** En nuestro estudio, la correlación negativa de Gas6 con IMC, CACS, CIMT y proteinuria, y los niveles más bajos de Gas6 en pacientes diabéticos sustentan la idea de que la disminución de los niveles de Gas6 en la insuficiencia renal crónica pueden jugar un papel en la calcificación vascular a través de la tolerancia alterada a la glucosa, la inflamación crónica, la disfunción endotelial y el aumento de la apoptosis. La importancia de nuestro estudio radica en que es el primero que muestra una relación entre la Gas6 y la proteinuria, la CACS y el CIMT en pacientes con insuficiencia renal crónica.

PALABRAS CLAVE: grosor íntima-media carotídeo; enfermedad renal crónica; puntaje de calcificación de arterias coronarias; proteína específica de detención del crecimiento 6; calcificación vascular

INTRODUCTION

Cardiovascular disease (CVD) is the most common cause of death in chronic renal failure (CRF), especially in hemodialysis patients. Vascular calcification is highly related with cardiovascular disease and frequently detected in CRF. The risk factors for VC are; increasing age, dialysis modality, secondary hyperparathyroidism, hyperphosphatemia, increased calcium-phosphate product, vitamin D treatment, diabetes mellitus, dyslipidemia and oral inhibitors of vitamin K.[1] Plain radiography and electron beam computed tomography for diagnosis of coronary artery calcification (CAC) have been developed for the detection and quantification of VC. An and Son have reported that significant VC on plain radio graphy was associated with CIMT, malnutri tion, inflammation and cardiovascular events in dialysis patients.[2] As known from the previous studies increased CIMT is an indicator of sub clinic atherosclerotic disease both in CKD and general population. CIMT measurement and CACS are commonly used in the early diagnoses of asymptomatic atherosclerotic disease.[3]

Growth arrest specific protein 6 (Gas6) is a member of vitamin-K dependent protein family. Gas6 is released from the lung, heart, kidney, bowel, endothelial cell, vascular smooth muscle cells (VSMC), bone marrow and monocytes.[4] Gas6 interacts with receptor tyrosine kinases of the TAM (Tyro3,Axl, Mer) family. The Gas6/Axl interaction plays important role in cell death, inflammation, glucose metabolism, protein synthesis, growth translation and proliferation. Gas6 is known to protect endothelial cells and VSMCs against apoptosis by inhibiting Bcl-2 induced Caspase 3 activation in apoptotic pathway.[5] It has been shown in recent studies that during phosphate-induced VSMC calcification, both Gas6 and Axl expressions are suppressed, resulting in downregulation of a survival signal, eventually promoting apoptosis and calcification.[6] Lee et al. have shown that the plasma Gas6 levels in patients undergoing coronary artery bypass grafting were significantly lower than control patients.[7] Zhao et al. showed a positive correlation between serum testosterone and Gas6 levels in male patients with CVD and both were significally lower than healty controls.[8]

The relationship between Gas6 and cardiovascular diseases has been demonstrated in many mouse models and cell cultures.

However, there are conflicting reports whether Gas6 levels are increasing or decreasing in human studies of diabetic and/or chronic renal failure. In present study the aim was to compare plasma Gas6 levels of CKD patients with control subjects and to examine the relationship of Gas6 with CIMT and CACS.

MATERIAL AND METHODS

Study design

This is a prospective, controlled and comparative study on 137 patients with chronic renal disease and 73 healthy controls.

Inclusion and exclusion criteria

Thirty-two chronic hemodialysis and 105 predialysis patients as well as 73 healthy controls

were enrolled in the study. Hemodialysis patients were undergoing 3 sessions per weekly. Each session lasted 4 hours and enoxiparin sodium 40 mg/session was used for each patient. Control group consist of individuals who have no history of cardiac disease and kidney failure as well as matched individuals in terms of age, gender.

All patients enrolled in the study were questioned for hypertension, hyperlipidemia and chronic diseases. Patients with documented atherosclerotic cardiac artery disease, periferic artery disease and related symptoms were not included in the study.Anticoagulated people were excluded from the study. Those individuals with suspected acute renal failure, pregnant women, exposure to contrast agent (in the past one month), malignancy and infectious symptoms were not included in the study.

Laboratory analyses

All patients' blood and urine samples were collected at 8 am after 12 hours of fasting. In dialysis patients blood samples were collected at midweek before the second dialysis session. Blood samples of Gas6 (Shanghai YL Biotech Co., Jiading District, Shanghai) were stored at -80^0 C and studied by ELISA. The readable range of the kit is 0.5-200 ng / mL and the sensitivity is 0.25 ng / mL. Because the reagent is a research reagent, it does not contain normal reference values for healthy people in its product data sheets.

Carotid intima-media thickness measurement

CIMT measurement was performed by the same radiologist. For all patients, one at the main carotid artery, one at the bulb level and one at the level of each of internal carotid artery, 3 bilaterally measurements were performed. As a whole total 18 measurements were performed for each patient and mean values were calculated. No measurement was performed where atherom plaque was seen.

Measurement of coronary artery calcification score

Measurement of CACS was performed by using multi-slice BT device. The amount of calcium in the coronary arteries was performed with the help of "Accu Image Workstation" and by using Agatston scoring. By adding calcium score in all coronary arteries total Agatston score was found.

Ethical approval: It was approved by the local Ethic Committee in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in Brazil 2013.

Statistical analyses

"SPSS for Windows version 15 Statistics" was used for data input and statistical analyses. Frequencies for categorized data type (qualitative) variations and standart deviation for continous data type (quantitative) variation was calculated as usual after data corrections comple ted, from pairwise comparisons p values were calculated; in the case of categorized data type variations were present, chi-square test, if one of variable was continous variable and distribution was appropriate t-test or one-way Anova parametric tests, if distribution was inapropriate non-parametric tests were used. If both varia bles have continous data, again considering dis tribution of variable, parametric (Pearson r) or non-parametric (Spearmen p) correlation tests were used. Statistical significance was determined as $p < 0.05$.

RESULTS

Patient characteristics

We enrolled 32 chronic hemodialysis patients, 105 predialysis chronic kidney disea se patients and 73 healthy subjects to stud-y groups. In predialysis group there were 23 patients (21.9%) at stage I, 23 patients (21.9%) at stage II, 30 patients (28.6%) at stage III, 17 patient (16.2%) at stage IV and 12 patients (11.4%) at stage V. In predialysis group hypertension frequency (78.1%) was found higher than control group (13%) and dialysis group (70.8%). Demographic characteristics of subjects were

depicted in **Table 1**. Since diabetes ratio was 41.1% in patient group, diabetes was the most common cause of renal failure as expected. The following etiologies were glomerulonephritis and hypertension. **(Table 1)**

Table 1. Demographic characteristics of subjects in the study groups

	Control Mean ±SD or % (n) (n:73)	Patients Mean ±SD or % (n) (N:137)		P value
Age (year)	53.26±9.04	55.04±15.09		0.687
Gender (M, %)	52.2	48.8		0.768
Hypertension	13.0	76.7		**<0.001**
Hyperlipidemia	21.7	34.1		0.242
Tobacco	56.5	42.6		0.218
SBP (mmHg)	122.95±11.74	131.28±18.71		0.007
DBP (mmHg)	77.34±9.04	76.56±11.29		0.716
BMI (kg/m^2)	25.81±4.30	27.50±5.30		0.104
Diabetes	4.3	41.1		**<0.001**
Etiology	-	Diabetes	34.1	
		Hypertension	24.8	
		Polycystic kidney	1.6	
		Post-renal diseases	2.3	
		Glomerulonephritis	32.6	
		Unknown	4.7	
Duration of renal failure (month)	_	28.57±38.42		

SBP: Systolic blood pressure, DBP:diastolic blood pressure, BMI: body mass index, SD: standart deviation

Laboratory parameters of subgroups

Serum creatinine was higher at predialysis and dialysis group than control. The hemoglobin and albumin levels were found significantly lower in dialysis and predialysis groups compared with the control. Fibrinogen and ferritin as inflammatory parameters and uric ascite, phosphate, Ca-P product and parathormone as atherosclerotic parameters were found significantly higher in both predialysis and dialysis groups compared to control. Comparative laboratory findings of all groups were shown in **Table 2**.

Gas6 level was 98.84±53.32 ng/mL at con trol group and statistically higher then dialysis and predialysis groups (p=0.001). 24 hours urine protein levels were significally higher at dialysis group than predialysis and control group. CIMT values were similar in predialysis and dialysis groups. These values were significan tly higher than control group. Although CACS was higher in dialysis group than predialysis and control group, the results were not statistically significant since the distribution range was very wide. **(Table 2)**

In patient group there were 56 diabetic and 81 non-diabetic patients. The Gas6 levels were lower in diabetic patients than non-diabetics. The CIMT and triglycerides levels were was significally increased in diabetics than non-diabetics. 10 patients were using calcium based phosphate binders and 9 patients were using vitamin D. There wasn't any difference between drug used and non-used groups.

Table 2. Comparative laboratory finding of all groups

	Control[c] Mean ±SD	Predialysis[p]	Dialysis[d]	P value	p values (Subgroup analyses)
WBC (10^3/μL) (4.5-11)	6441.30±1528.36	8170.70±2357.50	8654.55±2090.0	0.001	c<p, c<d, p=d
Hb (g/dL) (11.7-15.5)	14.17±1.69	11.84±2.15	9.81±1.19	0.001	c>p>d
Platelet (10^3/μL) (159-388)	232.78±47.19	268.08±96.64	239.25±58.35	0.107	
PT (INR) (0.8-1.2)	0.81±0.12	0.96±0.32	1.05±0.85	0.056	
Creatinine (mg/dL) (0.51-0.95)	0.78±0.16	1.96±1.64	5.94±1.55	0.001	**c<p<d**
Albumin (g/dL) (3.5-5.2)	4.29±0.39	3.67±0.85	3.28±0.76	0.001	c>p, c>d, p=d
Uric ascite (mg/dL) (2.6-6)	5.30±1.70	6.95±2.04	6.41±1.82	0.001	c<p, c=d, p=d
Fibrinogen (mg/dL) (200-393)	293.23±81.90	427.25±156.19	477.09±184.54	0.001	c<p, c<d, p=d
CRP (mg/dl) (0-5)	2.51±1.24	2.09±1.89	2.43±2.40	0.538	
Total cholesterol (mg/dl) (0-200)	195.73±37.41	220.44±66.9	228.5±128.74	0.292	
LDL (mg/dl)(0-130)	118.82±27.92	132.53±55.67	122.25±44.60	0.400	
TG (mg/dl)(0-150)	144.13±100.91	183.39±101.23	211.41±124.24	0.091	
HDL (mg/dl)(40-60)	47.60±11.20	51.37±17.47	45.05±10.77	0.165	
Calcium (mg/dl) (8.8-10.6)	9.40±0.60	9.27±0.80	8.51±1.41	0.060	
Phosphate (mg/dl) (2.5-4.5)	3.62±0.51	4.12±1.00	5.51±1.41	**0.001**	c<p, c<d, p=d
Ca-P product	34.14±5.61	37.95±8.72	49.14±13.40	**0.001**	**c=p, c<d, p<d**
MDRD-GFR (mL/min/1.73 m^2)	105.85±20.86	53.75±34.55	8.88±3.77	**0.001**	**c<p<d**
PTH (pg/ml) (12-88)	57.13±26.87	123.34±106.79	339.52±302.93	**0.001**	**c<p<d**
ALP (IU/L) (30-120)	74.90±26.16	108.23±99.77	105.17±56.84	**0.282**	
Ferritin (ng/dl) (11-306)	47.34±38.09	115.03±193.54	207.58±157.25	**0.008**	**c<d, p=d, c=p**
Proteinuria (mg/day)	62.15±147.80	1734.06±2539.12	3850.91±3892.16	**0.001**	c<p<d
Gas6 (ng/mL)	98.84±53.32	54.96±38.49	63.85±38.92	**0.001**	c>p, c>d, p=d
CIMT	0.46±0.10	0.66±0.17	0.65±0.19	**0.001**	c<p. c<d. p=d
CACS	0.13±0.62	259.40±775.10	452.68±900.83	0.111	

(WBC: White blood count, Hb:hemoglobin, PT: Protrombin time, CRP:C-reactive protein, LDL: Low density lipoprotein, TG: Triglycerides, HDL: High density Lipoprotein, MDRD-GFR: Modification of diet in renal Deseases Glomeruler filtration rate, PTH: parathormone, ALP: Alkaline phosphatase, Gas6: Growth arrest specific protein 6, CIMT: carotid intima media thickness, CACS: coronary artery calcification score)

Correlation analyses of CIMT, CACS and Gas6 values with laboratory parameters

Since control group also had traditional risk factors in terms of atherosclerotic disease, all groups were evaluated together and when distribution was expanded both CIMT and CACS were positively correlated with age, duration of CRF, creatinine, uric acid, fibrinogen, ferritin, proteinuria and each other and negatively correlated with Gas6 **(Table 3)**. In linear logistic regression analysis CIMT was significantly associated with age (β=0.473, p<0.001) and HDL cholesterol (β=-0.189, p=0.014).

The predictors for CACS were age (β=0.206, p<0.048) and proteinuria (β=0.281, p=0.002). In addition, negative correlation was detected between Gas6 and age, BMI, CACS, CIMT and proteinuria **(Table 3)**.

Table 3. The correlation of demographic and laboratory parameters with carotid IMT, CAC score and Gas6 levels

	CIMT rvalue	pvalue	CACS rvalue	p value	GAS6 rvalue	pvalue
Age	0.553	0.001	0.507	0.001	-0.179	0.032
Smoking years	0.205	0.012	0.107	0.193	0.073	0.385
CRF duration	0.170	0.037	0.230	0.005	-0.033	0.694
SBP	0.105	0.202	0.117	0.154	-0.049	0.561
DBP	-0.030	0.712	-0.105	0.200	-0.076	0.366
BMI	0.142	0.082	0.035	0.666	-0.223	0.007
Hemoglobin	-0.116	0.156	-0.215	0.008	0.038	0.650
Platelet	0.009	0.910	0.187	0.021	-0.054	0.521
PT (INR)	0.322	0.346	0.342	0.123	-0.078	0.465
Creatinine	0.227	0.005	0.268	0.001	0.043	0.609
Albumin	0.080	0.329	-0.160	0.050	0.044	0.604
Uric ascite	0.231	0.005	0.157	0.055	-0.086	0.310
Fibrinogen	0.351	0.001	0.310	0.001	-0.128	0.151
CRP	0.167	0.049	0.086	0.313	0.155	0.073
T cholesterol	0.000	0.996	0.065	0.433	0.166	0.049
LDL cholesterol	-0.003	0.967	0.049	0.551	-0.170	0.043
TG cholesterol	0.141	0.088	0.153	0.062	-0.127	0.131
HDL cholesterol	-0.257	0.002	-0.174	0.034	-0.070	0.409
Calcium	0.005	0.947	-0.096	0.245	-0.115	0.171
Phosphate	0.031	0.710	0.102	0.215	-0.045	0.599
MDRD-GFR	-0.266	0.001	-0.292	0.001	-0.027	0.752
PTH	0.126	0.130	0.080	0.340	0.070	0.413
ALP	0.079	0.343	0.073	0.380	-0.022	0.799
Ferritin	0.180	0.028	0.204	0.013	0.054	0.527
Proteinuria	0.221	0.007	0.226	0.005	-0.214	0.010
Gas6	-0.120	0.048	-0.227	0.006	-	-
CIMT	-	-	0.527	0.001	-0.120	0.048
CACS	0.527	0.001	-	-	-0.227	0.006

SBP: Systolic blood pressure, DBP:diastolic blood pressure, BMI: body mass index, SD: standart deviation, Hb:hemoglobin,PT: Protrombin time, CRP:C-reactive protein, LDL: Low density lipoprotein, TG: Triglycerides, HDL: High density Lipoprotein, MDRD-GFR: Modification of diet in renal Deseases Glomeruler filtration rate, PTH: parathormone, ALP: Alkaline phosphatase, Gas6: Growth arrest specific protein 6, CIMT: carotid intima media thickness, CACS: coronary artery calcification score

In the logistic regression analysis, Gas6 remained significantly associated with BMI (β=-0.224, p=0.005) and carotid IMT (β=-0.164, p=0.041) and proteinuria (β=-0.170, p=0.031).

In all CKD patients; statistically significant positive correlation was detected between Gas6 and creatinine (p=0.015) and PTH (p=0.005), negative correlation was detected between BMI (0.049), MDRD-GFR (p=0.009) and HDL cholesterol (p=0.009). In logistic regression model Gas6 significantly associated with BMI (β=-0.212, p=0.026). This results support that renal failure is only one of the factors, not the most important factor, effecting Gas6 levels.

DISCUSSION

In present study we have found decreased Gas6 levels in CKD patients and significant negative correlation between Gas6 and CIMT and CACS. Numerous studies have reported that CKD patients are candidate to medial calcification related with mineral metabolism disorders characterized by elevated serum phosphate levels and/or hypercalcemia.[9-11] Elevated extracellular calcium and phosphate levels affect the survival of VSMCs. Increased serum phosphate levels downregulated the expression of Gas6 and its receptor in VSMC.[12] This inhibition leads to the suppression of PI3K/Akt (phosphatidyl inositol-3 kinase/Akt) survival pathway and results as apoptosis in VSMC.[13] As mentioned above, the fact that high phosphate levels inhibit Gas6 expression underscores our findings that Gas6 levels were significantly lower in CKD patients than in controls. It is known that the function of Gas6 mainly depends on the vitamin K mediated glutamate carboxylation.[14] CKD patients especially hemodialysis (HD) patients have higher risk of vitamin K deficiency because of achieving a lower dietary load of potassium and phosphate, they limit foods including main sources of vitamin K.[15-16] Even significantly reduced vitamin K intake was also reported in a cohort of kidney transplant recipients with a median glomerular filtration rate of 61 ml/min, who did not have significant food intake limitations.[17] Jiang et al. investigated the effect of vitamin K2 on aortic calcification induced by warfarin via Gas6/Axl survival pathway in rats. They have found that warfarin decreased Gas6 and Axl and the expression of Bcl-2 protein levels. After 100μg/g vitamin K2 treatment Gas6, Axl and Bcl-2 expression were increased and calcification was reversed by 44%.[18] Higher phosphate and Ca-P product levels and vitamin K deficiency in CKD patients may explain the decreased Gas6 levels in our study.

The most important finding of the study was there was a negative relationship between Gas6 levels and CIMT, CACS and proteinuria. CIMT and CACS were known as predictors of early atherosclerosis. The carotid IMT and proteinuria were the predictors of Gas6 at regression analyses in the present study. There wasn't any study showing the relationship between Gas6 and CIMT and CACS in chronic renal failure in the literature. Proteinuria is an indicator of endothelial dysfunction in both diabetes mellitus and chronic renal failure and is considered to be equivalent to cardiovascular disease. Although the nature of relation between proteinuria and vascular disease is partly due to endothelial dysfunction, persistent low-grade inflammation also plays a role. Proteinuria as an inflammation marker and its relation with cardiovascular disease is usually neglected and ignored in hemodialysis patients. Trimarchi et al. have shown that proteinuria was seen 87% in 52 hemodialysis patients and nephrotic range proteinuria was significantly higher in diabetic nephropathy patients and was associated with inflammation and cardiac stress.[19] Endothelial dysfunction is one of the basic mechanisms in the atherosclerotic process among the causes of hypertension, diabetes, hyperlipidemia, homocysteinemia, cigarette smoking, obesity and advanced age. In a cross-sectional study of patients with type 2 diabetes with preserved kidney function, albuminuria was positively correlated with coronary and carotid artery calcification.[20] Li

et al. found that the Gas6 level was significantly decreased in diabetic patients with or without albuminuria compared to healthy controls and was further decreased in diabetic patients with microalbuminuria and macroalbuminuria compared to normoalbuminuria.[21] But there are conflicting results regarding proteinuria and Gas6 levels in diabetic patients. Erek-Toprak et al. showed that plasma Gas6 levels were higher in diabetic patients with micro or macroalbuminuria compared to diabetic patients with normoalbuminuria and healthy controls.[22] In our study, we have found CKD patients had lower levels of Gas6 than control group. Also Gas6 levels were lower in diabetic patients and CKD patients with proteinuria. Additionally there was a positive relation between proteinuria and CIMT and CACS. Proteinuria was also revealed as the predictor of CAC score in multiple regression analysis. Although CACS, another indicator of VC, was higher in dialysis group than predialysis and control group, the results were not statistically significant since the distribution range was very wide. Kristanto et al. indicated that the early onset of calcium deposition can remain invisible and Agatston score of zero does not always exclude the presence of incipient coronary calcification.[23] And cigarette smoking is about 56% in control group. May be for these reasons there wasn't statistical difference between CKD patients and controls but there was a negative relation between Gas6 and CACS in all group. In the literature, the relationship between Gas6 and VC in chronic renal failure was mostly studied in mice models and in vitro cell cultures.[24] There are three articles investigating the relation of Gas6 and VC in humans with chronic renal failure. Chen et al. showed that Gas6 levels were higher in hemodialysis patients than control group, and that this level was further increased with erythropoietin resistance related to inflammation and malnutrition.[25] Hallajzadeh et al. have found that Gas6 levels were higher in 46 hemodialysis patients than control group.[26] Lee et al. have shown that significantly increased Gas6 and protein S were found in CKD patients compared with controls and Gas6 levels were positively associated with low albumin and IV iron administration in hemodialysis patients.[27] Their results have shown that cumulative monthly dose of intravenous iron given on hemodialysis was moderately associated with higher Gas6 levels and in regression analysis only dialysis vintage and iron dosing were associated with Gas6 levels. This result supports that increased levels of Gas6 doesn't only related with CKD, iron treatment related oxygen reactive species and oxidative stress plays important role in Gas6 expression. Ciceri et al. found that phosphate challenge down-regulates the Gas6/Axl expression, and that Fe^{+3} treatment significantly prevents this downregulation in rat VSMC cultures.[28] In this study Ciceri et al. have shown that iron citrate inhibits high phosphate-induced Ca deposition by prevention of apoptosis, induction of autophagy, and partially affecting osteoblastic differentiation. Both in our study and Lee at al's even at lower levels of GFR there is a wide distribution of Gas6 levels, and some CKD patients have Gas6 levels that approach normal. Further studies are needed to determine why such different Gas6 levels can be seen at the same level of GFR. Both in our study and Lee at al's, because of the heterogeneous etiology of the CKD patients it is difficult to determine which disease conditions or nature of renal disease cause abnormal expression of Gas6. In our study, Gas6 levels were significantly lower in patients with CRF than control group. Although predialysis patients were higher than dialysis patients, it was not statistically significant. In CKD patients; statistically significant positive correlation was detected between Gas6 and creatinine and PTH, negative correlation was detected between BMI, MDRD-GFR. In logistic regression model Gas6 associated with only BMI. This results support that renal failure is only one of the factors, not the most important factor, effecting Gas6 levels.

All of these conflicting results in our study and literature show that there are different mechanisms in the regulation of Gas6 expression.

The choice of phosphate binder is one of the supporting finding. Non-calcium-based phosphate binders (NCBPB) are associated with decreased risk of all-cause mortality compared with calcium-based phosphate binders (CBPB) in patients with CKD.[29] There were preclinical data that demonstrate the ability of NCBPB, such as sevelamer and lanthanum to reduce in vitro VC.[30] Two new NCBPB are available to treat hyperphosphatemia in CKD, iron citrate and sucroferric oxyhydroxide. As mentioned above Ciceri et al. have found that phosphate challenge down-regulates the Gas6/Axl expression, and that Fe^{+3} treatment significantly prevents this downregulation in rat VSMC cultures.[28] We were using CBPB for most of our hyperphosphatemic patient.

There have been recent studies investigating the Gas6 levels in patients with coronary artery disease and/or cardiovascular risk factors. In a recent study Lee et al. have shown that the plasma Gas6 levels in patients undergoing coronary artery bypass grafting were significantly lower than control patients although serum creatinine levels were higher. Also there wasn't any correlation between creatinine and Gas6 levels. [7] In a study by Li et al. it was shown that Gas6 levels were lower in diabetic macroalbuminuric patients then controls although there was a moderate increase in creatinine levels and no relation was found between creatinine and Gas6 values.[16] Jiang et al. have shown that median plasma Gas6 levels were higher in healthy controls than patients with acute coronary syndrome.[31] Sunbul et al. showed that psoriasis patients with at least one cardiometabolic (CM) risk factor showed lower Gas6 levels compared to subjects without any CM risk factor.[32] It is well known that chronic renal failure patients have more than one cardiovascular risk factor.

In present study we have shown that Gas6 was negatively correlated with age. In a recent study including 589 healthy adults, Hung et al. have shown that Gas6 concentrations were inversely associated with age in both gender,[33] They have also shown that age in male and estrodiol in female were independent predictors of Gas6. It is not clear why such a relation occurs, perhaps the expression of Gas6 might be downregulated by aging process. A significant negative corelation between Gas6 levels and BMI was observed in our study. Kuo et al. found negative correlation between the plasma Gas6 levels and BMI, waist-to-hip ratio, HOMA- IR, IL-6 and E-selectin in women.[34] Li et al. have shown that Gas6 was inversely correlated with BMI and HbA1c in a study to investigate the diagnostic value of Gas6 in elderly patients with diabetic nephropathy.[21] As well as the underlying cause has not being fully understood, it can be explained by Gas6 and Axl gene polymorphisms and sex hormones and their association with adiposity, systemic inflammation and insulin resistance.

As a conclusion negative correlation between Gas6 and BMI, carotid IMT, CACS and proteinuria and lower Gas6 levels in diabetic patients support that decreased Gas6 levels in chronic renal failure may have a role in vascular calcification through altered glucose tolerance, chronic inflammation, endothelial dysfunction and increased apoptosis. The underlying causes of decreased Gas6 can be vitamin K deficiency, high phosphate and Ca-P product levels and usage of calcium based phosphate binders. Our study has an importance because it is the first study showing a relation between Gas6 and proteinuria, CACS and carotid IMT in patients with chronic renal failure. Considering that the Gas6 / Axl system is involved in many different pathways, new comprehensive and high patient number of studies are needed to explain these contradictory results in the literature. Therapeutic strategies targeting the Gas6 / Axl system may be a signal for patients with chronic renal failure which cardiovascular disease is the most important death cause.

Our study has two main limitations. First, many different glomerular diseases and other causes of chronic renal disease evaluated together, the sample wasn't homogeneous and sample size was relatively small. Another limitation is the lack of markers related with inflammation

and atherosclerosis, for example homocysteine. Prospective studies with higher number of patients and homogenous subgroups are needed to assess the relation.

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