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Levels of arginine and its products in dialysis patients

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Abstract. – OBJECTIVES: Chronic renal failure is among significant public health problems all over the world. Despite advances in diagnosis and treatment approaches, cardiovascular morbidity and mortality is still high in chronic renal failure patients on dialysis. The aim this study is to investigate the importance of arginine, asymmetric dimethylarginine (ADMA) and nitric oxide (NO) levels in the pathophysiology of cardiovascular morbidity and mortality in patients with dialysis treatment program.

PATIENTS AND METHODS: 53 patients with chronic renal failure receiving hemodialysis treatment and 34 healthy persons were participated to the study. Arginine and ADMA levels were measured by high-performance liquid chromatography (HPLC) fluorescence detector. NO levels were assessed by a colorimetric method. Albumin, urea, creatinine levels was performed using the spectrophotometric method.

RESULTS: Arginine levels were similar in dialysis patients when compared to the control group. Similarly NO levels did not show any difference between patient group and the control group. ADMA levels were found to be significantly high in dialysis patients compared to the control group ($p < 0.005$). Arginine/ADMA ratio was lower in the patient group compared to the control group ($p < 0.001$). Dialysis patients who have diagnosed coronary artery disease had low levels of albumin and creatinine. Arginine levels, ADMA levels and NO levels did not show any difference in the patients with coronary artery disease or not ($p > 0.05$). Arginine levels were significantly higher in men compared to women. Pearson correlation analysis showed that there was a correlation between NO and arginine levels. Arginine and Arginine/ADMA showed a positive correlation while ADMA and arginine/ADMA levels showed a negative correlation.

CONCLUSIONS: As a result, our data showed that ADMA clearance was impaired in hemodialysis patients. Increase in ADMA levels may play a role in atherosclerosis dependent morbidity and mortality.

Key Words:
Arginine, Asymmetric dimethylarginine, Atherosclerosis, Hemodialysis, Nitric oxide.

Introduction

Chronic renal disease (CRD) is among significant public health problems all over the world. Despite advances in diagnosis and treatment approaches, cardiovascular morbidity and mortality is still high in chronic renal failure patients on dialysis. The leading causes of morbidity and mortality of CRD patients are cardiovascular diseases such as atherosclerosis, cardiac insufficiency and myocardial infarction[1,2].

In CRD, besides common and CRD specific risk factors, the role of a number of molecules associated with cardiac diseases and accelerated atherosclerosis have been identified. Among these molecules, the recently identified ones are homocysteine, C-reactive protein, interleukin-6, intracellular adhesion molecule (ICAM-1), arginine products, asymmetric dimethyl arginine (ADMA), cardiac specific troponin-I have been identifed[1,3-7].

ADMA is accepted to be a risk factor for the development of coronary artery disease by causing endothelial dysfunction and vasospasm with inhibition of local nitric oxide (NO) synthesis[8]. ADMA levels have been reported to be increased in renal failure, heart failure, coronary artery disease, hypertension, hypercholesterolemia, hyperhomocysteinemia and diabetes mellitus[9,10].

Furthermore CRD increase the risk of cardiovascular diseases and related complications by altering levels of a number of arginine products such as ADMA and NO[1,6].

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The aim this study is to investigate the importance of Arginine, ADMA and NO levels in the pathophysiology of cardiovascular morbidity and mortality in patients with dialysis treatment program.

Patients and Methods

Study Population

As the first step, Selçuk University Human Studies Ethics Committee approval was obtained (2006/095) for the study. Among all dialysis patients (n = 53; 33 male, 20 female) in Konya-Karaman region those agreed to participate, based on obtained informed consent, were included in the study. A control group (n = 34) comprising of healthy volunteers was created. The inclusion criteria were; being older than eighteen years of age, experiencing at least three month-period of dialysis treatment program, giving informed consent, being able to communicate, and having full civil capacity and normal cognitive functions.

Obtaining Samples and Storage

Two separate venous blood samples were taken to plain tubes with gel prior to dialysis, from each subject. Blood samples were immediately transferred to the laboratory in accordance with cold chain maintenance. Samples were centrifuged at 4°C for 10 minutes at 2000 × *g*, using refrigerated centrifuge. For ADMA and arginine analyses 2 mL of serum transferred to eppendorf tubes after deproteinization with sulfosalicylic acid and were stored at -80°C until analysis.

Albumin, Urea and Creatinine Measurements

Beckman Coulter LX20 auto-analyzer was used for the measurements. Spectrophotometric assay was performed using Beckman kits and results were calculated as g/dL.

Measurement of Nitric Oxide Levels

Nitric oxide levels were analyzed by Griess reaction described by Cortas and Wakid[11]. Total nitrite (nitrate + nitrite) concentration was evaluated by colorimetric kit method (Cayman Chemical Company, CM780001, USA). Auto-analyzer measurements were recorded at 490 nm and results were expressed as µmol/L.

Measurement of Arginine and ADMA Levels

Agilent 1100 HPLC was used for measurement of arginine and ADMA levels. Appropriate column, mobile phase conditions for the HPLC analysis were provided.

Mobile Phase Preparation

Firstly 170 mL of methanol was added to 820 mL of sodium acetate buffer (pH 6.8). Afterwards, 10 ml of tetrahydrofuran was added to this solution and 1 L of mobile phase A was prepared. In order to prepare 1 L of mobile phase B 770 mL of methanol and 10 ml of tetrahydrofuran were added to 220 mL of sodium acetate buffer (pH 6.8). Degassing mobile phases was performed by filtration through a 0.45 µm membrane.

Standard Solutions

Stock solutions of 1 mM of arginine were prepared within 0.1 M HCl in different concentrations as 600 µM, 300 µM, 150 µM, 75 µM, 37.5 µM and 18.75 µM. After derivatization, standard solutions placed in automatic sampling device and 10 µl injected. Arginine standard chart was created using obtained peak areas. Standard ADMA solutions at concentrations 25 µM, 12.5 µM, 6.25 µM, 3.1 µM, 1.56 µM and 0.78 µM were prepared from 0.5 mM of ADMA stock solution within 0.1 M HCl. After derivatization process these standard solutions placed in automatic sampling device and injected into HPLC. ADMA standard chart was created using obtained peak areas.

Derivatization and Standart Charts

Standard solutions and samples were derivatized using o-phthalaldehyde (OPA). As derivatization solution, 10 mg OPA was dissolved within 0.5 ml methanol, and 2 ml of a 0.4 mM borate buffer. The pH of solution was set to 10 and 30 µL mercaptoethanol was added to freshly prepared derivatization solution. Afterwards, 1.3 µL of the sample supernatant was added to 8.7 µL derivatization solution and applied to device 3 minutes later. Standard graphs were prepared using areas obtained peaks at the end of analyses. Arginine and ADMA levels were calculated utilizing these graphs.

Chromatography

Using Agilent 1100 HPLC, fluorescence detector was measured at excitation and emission

wavelengths of 338 and 425 nm, respectively. Chromatography was performed on a 250 x 4.6 mm C_{18} Supelcosil 5 µm column. Total analysis time set to 29 minutes. Gradient mobile phase was used. Mobile phase A and B was pumped to the system 1 ml per minute. Retention time for arginine was 17.9 minutes and calibration graph was obtained by analyzing standard values. Retention time for ADMA was 18.7 minutes and calibration graph was obtained by analyzing standard values.

Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences software (SPSS 16.0 for Windows). Since data does not fulfill normal distribution non-parametric Mann-Whitney U test was used. Pearson's correlation tests were used to analyze the relationship of parameters. A p value of < 0.05 was considered significant.

Results

Study Population

The study of consisted of 53 chronic renal failure patients (33 male, 20 female) undergoing hemodialysis treatment and 34 healthy individuals as control group. The mean age of male patients was 56.67 ± 15.64 and was 55.00 ± 10.67 for females. Age, sex, duration of dialysis and predialysis urea, creatinine, and albumin levels of patients and control group is shown in Table I. As seen in the table significant difference was observed between urea, creatinine, and albumin levels of patients and the controls.

Levels of Arginine, ADMA and NO

Average arginine, ADMA and NO levels of dialysis patients and controls are shown in Table II.

Average arginine level in the control group was 399.3 ± 193.0 µmol/L, while it was 355.8 ± 207.9 µmol/L in dialysis patients. However arginine level in dialysis patients was lower, no statistically significant difference was observed.

Average ADMA level was 2.7 ± 1.4 µmol/L in the control group and it was 7.3 ± 4.8 µmol/L in the patient group. ADMA levels were found to be significantly higher in dialysis patients ($p < 0.005$).

Arginine/ADMA ratio was statistically significantly lower in the patient group (52.8 ± 32.9) compared to control group (148.4 ± 85.3) ($p < 0.001$).

The average serum nitric oxide level in dialysis patients was 4.96 ± 3.10 µmol/L, while it was 4.89 ± 2.67 µmol/L in the control group. Serum nitric oxide levels in dialysis patients were slightly higher than the controls, however, this difference was not statistically significant ($p > 0.05$).

ADMA levels of dialysis patients and controls are shown in Figure 1.

Arginine levels of dialysis patients and controls are shown in Figure 2.

Table I. General parameters of patients and controls.

Parameters	Patients	Controls	p
n (Male/Female)	53 (33/20)	34 (19/15)	
Age (year)	56.04 ± 13.88	50.80 ± 17.41	NS
Urea (mg/dL)	101.65 ± 17.02	29.42 ± 6.00	$p < 0.05$
Creatinine (mg/dL)	8.76 ± 1.92	1.05 ± 0.12	$p < 0.05$
Albumin (g/dL)	3.72 ± 0.30	4.56 ± 0.52	$p < 0.05$
Dialysis duration (month)	40.09 ± 35.92	–	

NS: Not significant.

Table II. Average arginine, ADMA and NO levels of dialysis patients and controls.

Parameters	Patients	Controls	p
n	53	34	
ADMA (µmol/L)	7.3 ± 4.8	2.7 ± 1.4	$p < 0.005$
Arginine (µmol/L)	355.8 ± 207.9	399.3 ± 193	NS
Arginine/ADMA	52.8 ± 32.9	148.4 ± 85.3	$p < 0.001$
NO (µmol/L))	4.89 ± 2.67	4.96 ± 3.10	NS

NS: Not significant.

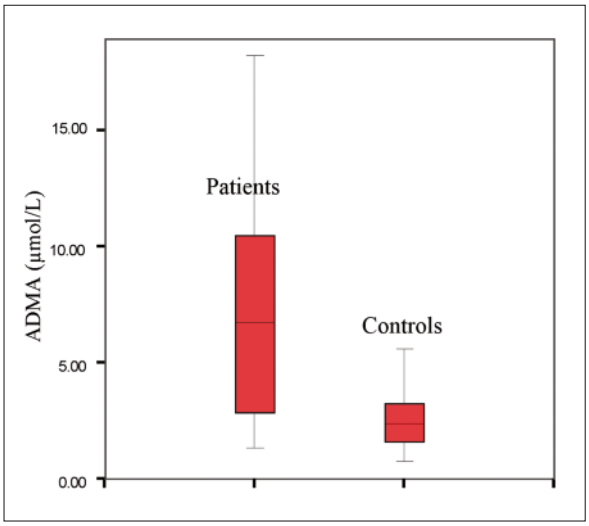

Figure 1. ADMA levels of dialysis patients and controls.

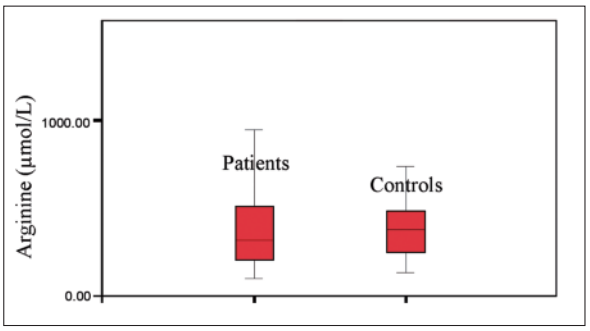

Figure 2. Arginine levels of dialysis patients and controls.

Arginine/ADMA ratios of dialysis patients and controls are shown in Figure 3.

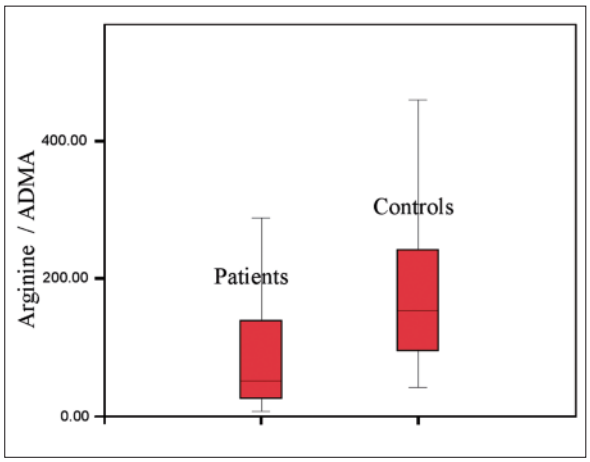

Figure 3. Arginine / ADMA ratios of dialysis patients and controls.

Evaluation Dialysis Patients in Terms of Coronary Artery Disease

Examination, medical history, ECG and other laboratory findings of 53 dialysis patients revealed that 13 had diagnosis of coronary artery disease (CAD) previously. However, remaining was free from ischemic cardiac or coronary artery diseases. The relationship of coronary artery disease and parameters of dialysis patients is shown in Table III.

No statistically significant difference was observed between serum arginine, ADMA and NO levels of dialysis patients with and without coronary artery disease.

Average albumin level of dialysis patients with CAD was 3.56 ± 0.25, while it was 3.78 ± 0.30 in patients without coronary artery disease. This difference was statistically significant (p = 0.019).

Average duration of dialysis was 22.6 ± 18.9 months in patients with coronary artery disease, while the average duration was 45.8 ± 38.4 months in patients without coronary artery disease. Duration of dialysis was significantly higher in patients without coronary artery disease (p = 0.035).

Correlation Analysis of Dialysis Patients

Pearson's correlation analysis between the levels of arginine, NO, ADMA and arginine/ADMA ratio in patients with long-term dialysis treatment is shown in Table IV.

Positive and significant correlation was observed between Arginine and NO levels in terms of the length of duration of dialysis. Furthermore, positive correlation was observed between Arginine levels and Arginine/ADMA ratio in terms of the length of duration of dialysis. However, negative correlation was observed between ADMA levels and Arginine/ADMA ratio in terms of the length of duration of dialysis.

Table III. The relationship of coronary artery disease and parameters of dialysis patients.

Parameters	Patients with CAD	Patients without CAD	*p*
Number	13	40	
Age	65.15 ± 7.64	53.08 ± 14.23	p = 0.002
Urea (mg/dL)	103.8 ± 18.9	106.6 ± 17.3	NS
Creatinine (mg/dL)	8.3 ± 1.9	8.9 ± 1.9	NS
Albumin (gr/dL)	3.56 ± 0.25	3.78 ± 0.30	p = 0.019
Arginine (µM)	354.5 ± 199.9	356.2 ± 213.0	NS
ADMA (µM)	5.50 ± 3.55	7.92 ± 5.03	NS
NO (µM)	4.50 ± 2.42	5.02 ± 2.76	NS
Dialysis duration (month)	22.6 ± 18.9	45.8 ± 38.4	p = 0.035

NS: Not significant; CAD: Coronary artery disease.

Table IV. Pearson's correlation analysis between the levels of arginine, NO, ADMA and arginine/ADMA ratio in patients with long-term dialysis treatment.

Parameters n = 53)	NO	ADMA	Arg/ADMA
Arginine	$p = 0.012$ r = 0.34*	$p = 0.171$ r = 0.203	$p = 0.010$ r = 0.374*
NO	–	$p = 0.875$ r = -0.024	$p = 0.838$ r = 0.031
ADMA	–	–	$p = 0.0001$ r = -0.583**

*Significant correlation at $p < 0.05$; **Significant correlation at $p < 0.0001$.

Pearson's correlation analysis between age, duration of dialysis, urea, creatinine, arginine and NO levels of dialysis patients is shown in Table V.

There were negative and significant correlation ($p = 0.001$, r = -0.43) between age of dialysis patients and their serum albumin levels. Furthermore, positive and significant relationship ($p = 0.014$ r = 0.34) was detected between duration of dialysis and their serum albumin levels.

Statistically significant positive correlation was observed between urea and albumin levels in dialysis patients ($p = 0.001$, r = 0.45). Additionally, there were significant positive correlation between urea and creatinine levels ($p = 0.0001$, r = 0.81), as well.

Similarly, statistically significant positive correlation was found between albumin and creatinine levels in dialysis patients ($p = 0.0001$, r = 0.50). Statistically significant positive correlation was observed between arginine and nitric oxide levels ($p = 0.012$, r = 0.34), as well. There were significant positive correlation between NO and creatinine levels ($p = 0.006$, r = 0.37) in dialysis patients.

Table V. Pearson's correlation analysis between age, duration of dialysis, urea, creatinine, arginine and NO levels of dialysis patients.

Dialysis patients (n=53)	Albumin	Creatinine	NO
Age	$p = 0.001$ r = -0.43**	–	–
Dialysis duration (months)	$p = 0.014$ r = 0.34*	–	–
Urea (mg/dL)	$p = 0.001$ r = 0.45**	$p = 0.0001$ r = 0.81***	–
Creatinine (mg/dL)	$p = 0.0001$ r = 0.50***	–	–
Arginine (µM)	–	–	$p = 0.012$ r = 0.34*
NO (µM)	–	$p = 0.006$ r = 0.37**	–

*Significance correlation at $p < 0.05$; **Significance correlation at $p < 0.005$; ***Significance correlation at $p < 0.0001$.

Discussion

In this study, serum ADMA levels were considerably high in patients with stage V CRD undergoing dialysis treatment. However, previously conducted studies indicate ADMA as a potential risk factor for cardiovascular disease, there is still not meta-analysis of randomized controlled clinical trials dealing with the relationship between ADMA and cardiovascular risk. Besides ADMA interactions protein methylation, dimethylarginine dimethylamonohydrolase (DDAH) and NO pathways possibly contribute to the pathophysiology of cardiovascular disease[12].

In dialysis patients, reduced glomerular filtration rate is responsible for reduced ADMA molecule elimination. Higher levels of ADMA in dialysis patients compared to the healthy controls might be attributed to impaired renal function and decreased activity of DDAH enzyme that is effective in catabolism of ADMA. In compatible with the present study, previously conducted studies have also indicated high ADMA levels in dialysis patients[13-15].

The literature has previously indicated the relationship between endothelial dysfunction and increased ADMA levels in dialysis patients[16]. Therefore, increased ADMA might be named among risk factors responsible for endothelial dysfunction, cardiovascular diseases and mortality in hemodialysis patients. ADMA is eliminated by dialysis treatment; however, it rises again after hemodialysis[17]. Moreover, the study by Abedini et al. has showed the role of high ADMA levels in increased morbidity, and mortality in patients with chronic renal failure, besides contribution of functional deterioration of graft in renal transplant recipients[7].

Glassock and Rule showed decreased blood flow of liver, kidney and spleen in association with deteriorated systemic hemodynamics due to decreased Arginine and increased ADMA levels. The same study also indicated reduced NO production as an important cause at initial phase of organ failure[18]. In the present study, serum NO

levels in dialysis patients were slightly higher than the controls, however, this difference was not statistically significant ($p > 0.05$). This might be caused by labile molecular structure of NO, influence of certain compensatory mechanisms or analysis/procedure-related.

In this work, serum arginine levels in dialysis patients were slightly lower than the controls; however, this difference was not statistically significant ($p > 0.05$).

In accordance with the literature, the present study also indicated increased ADMA levels in end stage CRD patients. Among cardiovascular disease related risk factors ADMA should not be neglected besides commonly known risk factors. This molecule is of high clinical importance in terms of long term prognosis of CRD patients. The incidence of cardiovascular events in this population might be decreased by reducing ADMA levels. In this regard, there is an increasing need for studies regarding reducing ADMA levels. An important cause of high cardiac mortality of dialysis patients is high prevalence of cardiac diseases in patients showing hemodialysis indications. In a study by Cheung et al. out of 1846 dialysis patients 80% had cardiac diseases, at onset of treatment[19]. On the contrary in our series, examination, medical history, ECG and other laboratory findings of 53 dialysis patients revealed that 13 had diagnosis of coronary artery disease (CAD) previously. However, remaining was free from ischemic cardiac or coronary artery diseases.

In this research, average duration of dialysis was significantly higher in patients without coronary artery disease. This might be caused by short-term survival in patients with cardiovascular diseases.

The relationship of serum albumin level and morbidity and mortality of hemodialysis patients has been clearly established in the literature. The risk of death in dialysis patients showing serum albumin levels lower than 2.5 g /dL is reported to be twenty-fold compared to those with serum albumin levels over 4 g/dL[20]. In this study, on the other hand average albumin level of dialysis patients with CAD was significantly lower compared to without CAD. Serum albumin levels gets lower with advancing age, which might be responsible for increasing incidence of CAD in older ages.

Significant changes are observed in serum amino acid concentrations because of the reduction in the rate of protein synthesis secondary to decrease in glomerular filtration rate[21]. In our study, a statistically significant increase was observed in urea and creatinine levels in hemodialysis patients (for both $p < 0.05$), while serum albumin levels were significantly lower in the dialysis patients compared to the control group ($p < 0.05$). The metabolism of protein, of albumin in particular, affects the prognosis in the course of this CRD. Moreover, decreased rate of synthesis of albumin increases the risk of cardiovascular disease and mortality[22].

Examination of dialysis patients in terms of gender; positive correlation between albumin levels and duration of dialysis treatment especially in female patients suggests higher levels of albumin in hemodialysis patients results long-term survival. We also determined a positive correlation between higher levels of albumin, and levels urea and creatinine in female patients. On the other hand, positive correlation between creatinine and NO levels in this patient group might be anticipated as contributive factor for lower risk of cardiovascular morbidity and mortality. Therefore, high level of albumin in female dialysis patients may be considered as a factor prolonging the survival during dialysis treatment. In male patients at older ages, the negative correlation between serum albumin levels and patients' age indicates the higher risk of cardiovascular disease and mortality.

In chronic hemodialysis patients, determination of levels of arginine and its analogs, and to find out intervening variables is of high importance. Arginine is located in structures of important compounds in various tissues. Arginine takes place in a number of metabolic pathway such as protein synthesis, glucose and glycogen formation, ornithine, urea, NO, creatinine, agmatine, proline and glutamate synthesis, as well as biosynthesis of polyamines and also joining the structures of tuftsin and ADH[23,24].

Among dialysis patients the levels of arginine and creatinine were significantly higher in males compared to female patients. This finding was remarkable. However, male patients have more muscle mass, this difference should be clinically examined.

Slight decrease in plasma arginine levels of end stage renal failure patients might be caused by deterioration of the arginine transport and metabolism. Plasma arginine levels do not reflect the intracellular pool. The literature has previously indicated abnormalities in amino acid metabolism in dialysis patients[25].

NO, a molecule synthesized from L-arginine, is known to be vasoprotective since it enhances endothelial cell survival and proliferation, inhibits the excessive proliferation of vascular smooth muscle cells, and suppresses the adhesion of platelets and inflammatory cells to the vessel wall[26,27].

In the present work, NO levels were slightly lower in dialysis patients compared to healthy controls, however, this difference was not statistically significant. This might be caused by insufficient amount of arginine, increased amount of inhibitors or passage of the molecule to dialysate solution during hemodialysis.

ADMA is an endogenous inhibitor of NO synthase (NOS). It causes endothelial dysfunction by reversibly blocking NO synthesis from L-arginine, and consequently atherosclerotic vascular disease may be initiated or aggravated (3). In the present study, ADMA levels were significantly higher in dialysis patients compared to controls. A number of prospective clinical studies have supported the relationship of high ADMA concentrations and increased cardiovascular risk. On the other hand, the relationship ADMA and NOS insufficiency remains controversial[28].

We have found the ratio of arginine levels to ADMA levels to be significantly higher in hemodialysis patients, which might suggest the importance of use of arginine/ADMA ratio in terms of assessment of the severity of kidney damage. Negative correlation was found between ADMA levels and increasing age in female dialysis patients. The risk of cardiovascular disease increases with age; however, lower ADMA levels and long dialysis treatment duration might result in long-term survival in this patient group.

Today, unfortunately mortality rate is still quite high in patients with end-stage CRD that is even worse than certain types of cancer. The more close serum albumin and arginine levels to normal values the longer survival is seen in dialysis patients. High levels of serum urea and creatinine during dialysis treatment is usually interpreted incorrectly. However, besides high urea and creatinine levels, a long survival is expected for patients who gains quality protein with appropriate nutrition[29].

A number of serious problems have been identified for end-stage CRD patients on dialysis treatment. More detailed clinical researches should be implemented in order to develop methods that would increase the effectiveness of dialysis treatments and to find out further preventive treatment methods for cardiovascular health.

Conclusions

To understand the pathophysiology of cardiovascular disease in dialysis patients, besides widely known classical risk factors, changes in protein metabolism, arginine metabolism in particular, should be investigated more extensively. The comparison of progressive changes of arginine and its products in dialysis patients to other disease groups might be helpful for further evaluations. Additionally investigation of more related variables will also contribute understanding this issue. Moreover, based on obtained findings considering arginine and its metabolites in terms of assessment of morbidity and mortality of cardiovascular disease, at all stages of CRD, is recommended.

ADMA is considered as an important parameter to play role in vasoconstriction dependent endothelial dysfunction, increased blood pressure and atherosclerosis, and related complications. Therefore it has clinical importance in the prognosis of patients with CRD. The incidence of cardiovascular events in this population might be decreased by reducing ADMA levels. In this regard, there is an increasing need for studies regarding reducing ADMA levels.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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