

Serum Fetuin-A in Chronic Renal Disease Patients: Contribution to Endothelial Dysfunction and Hemostatic alteration

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Abstract: Background/Aim: Fetuin-A is a circulating calcium-regulatory glycoprotein that inhibits vascular calcification. In the present study, serum fetuin-A was studied as a novel risk factor for the development of endothelial dysfunction (ED) and hemostatic alteration in patients with chronic renal disease (CRD). **Patients and Methods:** 15 CRD patients on conservative treatment, 15 end stage renal disease (ESRD) patients on regular hemodialysis (HD) treatment and 15 healthy volunteers were enrolled in the study. Fetuin-A, thrombomodulin (TM), von Willebrand factor (vWF), tissue plasminogen activator (t-PA), plasminogen activator inhibitor (PAI-1), D-dimer, high sensitivity CRP (hs CRP) and IL-6 were measured by ELISA. **Results:** There was a significant reduction in Fetuin-A levels in CRD and HD patients compared to controls. A significant decrease was also detected in HD group when compared to CRD group. The inflammatory markers, hs CRP and IL-6, were significantly increased in CRD and HD patients in comparison to controls. The increase was also significant on comparing HD group to CRD group. A strong inverse correlation was found between serum fetuin-A and each of hs CRP and IL-6. In addition, regression analysis revealed that hs CRP is an independent determinant of serum fetuin-A level. The traditional markers of ED, TM and vWF, were significantly increased in CRD and HD patients compared to controls. The increase was also significant when HD patients were compared to CRD patients. The significant inverse correlation between fetuin-A and each of TM and vWF supports the hypothesis that low serum fetuin-A with subsequent vascular calcification could be one of the contributing factors for the development of ED in CKD and HD patients. The fibrinolytic parameters tPA, PAI-1 and D-dimer levels were significantly higher in CRD and HD compared to controls. HD patients had significantly higher values of the previously mentioned parameters in comparison to CRD patients. t-PA, PAI-1 and D-dimer were significantly correlated to fetuin-A in CRD and HD patients. **Conclusion:** The results of this study demonstrate that in CKD and HD patients inflammatory processes are increased and linked to low fetuin-A and vascular calcification which represents a novel risk factor for the development of ED. The interplay of these phenomena could be responsible for the development and progression of accelerated thrombogenesis that is peculiar to renal patients.

[Nadia A. Hussein, Ola M. Mahmoud, Manal Y. Zahran, Manar A. Rafaat. **Serum Fetuin-A in Chronic Renal Disease Patients: Contribution to Endothelial Dysfunction and Hemostatic alteration.** Journal of American Science 2010;6(12):1098-1105]. (ISSN: 1545-1003). <http://www.americanscience.org>.

Keywords: Serum Fetuin; Chronic Renal Disease; Patients; Endothelial Dysfunction; Hemostatic alteration

Introduction:

Hemostasis is a process of blood clot formation at site of vessel injury. When a blood vessel wall breaks, the hemostatic response must be quick, localized and carefully regulated. Bleeding or thrombosis may occur due to missing or dysfunctional moieties of the coagulation or fibrinolytic factors [1]. Disturbances in hemostasis are common complications of chronic renal disease (CRD). Their occurrence and severity correlate quite well with the progressive loss of renal function to end stage renal disease (ESRD) [2]. The association of CRD with thrombotic events is somewhat puzzling because renal disease is typically associated with increased bleeding tendency due to platelet

dysfunction and disturbed plasma coagulation [3]. However, recent studies have indicated that CRD is associated with an impaired function of the vascular endothelium [4]. Profound endothelial dysfunction is a prominent pathologic feature of uraemia [5,6]. Endothelial cell injury is the probable cause due to uremic toxins retention, dyslipidemia, hypertension and secondary hyperparathyroidism as well as increased levels of interleukin-1 (IL-1) and tumor necrosis factor- α (TNF- α) [1]. If such a defect also involves the endothelial fibrinolytic system, it may provide a potential mechanism of reduced thromboresistance. It is probable that disturbances in fibrinolytic activity and endothelial dysfunction may play a role in vascular complications such as stroke or ischemic heart disease [7] which represent the

leading causes of morbidity and mortality in CRD [8].

Fetuin-A, a circulating calcium-regulatory glycoprotein that inhibits vascular calcification, is predominantly synthesized in liver. It is secreted into the blood stream and deposited as a noncollagenous protein in mineralized bones and teeth. During fetal life, there is high serum concentration of fetuin-A. Its level declines following infection, acute or chronic inflammatory states and malignancy [9,10]. Low serum fetuin-A levels have been reported in CRD and dialysis patients and is associated with inflammation and outcome [11,12]. Recent data have shown a relationship between vascular calcification and endothelial dysfunction (ED) in vascular disease [13]. Therefore, low serum fetuin-A level could be one of the contributing factors for the development of ED in CRD patients.

This study was designed to unveil the possible role of fetuin-A in the development of ED and hemostatic alteration in CRD patients.

Subject and Methods

Study population

The study was conducted on 30 patients admitted to Nephrology Department and Dialysis unit (Theodor Bilharz Research Institute). The patients were divided into two groups:

Group A: This included 15 CRD patients on conservative treatment (8 males and 7 females, ages ranging between 19-70 years with a mean of 44 ± 14.9).

Group B: This included 15 ESRD patients (9 males and 6 females with ages ranging between 33-65 years with a mean of 52 ± 9.9) on regular hemodialysis (HD) treatment (3 sessions weekly, 4 hours each for a period of more than 3 months) using Fresenius 4008 B machine, Hemophane filters with 1.4 surface area and sodium acetate solution as a dialyzate.

The etiology of CRD was variable between the 2 studied patient groups (hypertension, diabetes mellitus, urologic and unknown causes). None of the patients received blood or blood components transfusion therapy in the past 21 days and none of them was receiving erythropoietin therapy. This study was approved by the ethical committee of Theodor Bilharz Research Institute. Informed consents were obtained from all patients in accordance with the Declaration of Helsinki.

Fifteen age and sex matched healthy subjects selected from medical and paramedical staffs were included in the study to serve as a control group.

Sampling

A sample of 6 ml blood was collected from each subject into sterile endotoxin-free vacuum blood collection tubes, of which 2 ml were collected on potassium EDTA for hemogram by automated hematology analyzer ACT Differential (Beckman Coulter, France), 2 ml on trisodium citrate to measure thrombomodulin (TM), von Willebrand factor (vWF), tissue plasminogen activator (t-PA), plasminogen activator inhibitor (PAI-1) and D-dimer levels. In addition, 2 ml were withdrawn into a plain tube and left to clot. Sera obtained were collected for kidney function tests using standard methods. The rest of the sera were aliquoted, stored and kept frozen at -70° to measure the serum levels of fetuin-A, high sensitivity CRP (hs CRP) and IL-6.

Assay methods

Quantitative analysis of fetuin-A:

Serum fetuin-A level was measured by ELISA technique using human fetuin-A kit (BioVendor, France).

Quantitative analysis of inflammatory markers:

- High sensitivity CRP (hs CRP) was assayed by ELISA technique using hs CRP kit (BioVendor, France).
- Interleukin 6 (IL-6) was measured by ELISA technique using human IL-6 kit (BioSource, USA)

Quantitative analysis of endothelial and fibrinolytic markers:

- Plasma thrombomodulin (TM) level was assayed by ELISA technique using Asserachrom TM kit (Diagnostica Stago, France).
- Plasma von Willebrand factor (vWF) antigen level was assayed by ELISA technique using an Asserachrom vWF kit (Diagnostica Stago, France).
- Plasma level of tissue plasminogen activator (t-PA) antigen was assayed by ELISA technique using ZYMUTEST t-PA antigen kit (Hyphen BioMed, France).
- Plasma level of plasminogen activator inhibitor (PAI-1) antigen was assayed by

ELISA technique using ZYMUTEST PAI-1 antigen kit (Hyphen BioMed, France).

- o Plasma D-dimer level was assayed by ELISA technique using ZYMUTEST D-dimer kit (Hyphen BioMed, France).

Statistical analysis

Statistical analysis was done by a statistical software package (SPSS 16.0 for Microsoft windows, SPSS Inc.). The levels of studied parameters were analyzed by Mann-Whitney test. Data were expressed as arithmetic mean \pm standard deviation. Pearson correlation coefficient 'r' was used to measure the relationship between two variables. Stepwise multiple regression analysis was employed to evaluate any association between the serum level of

fetuin-A and markers of endothelial dysfunction, inflammation and fibrinolysis markers tested. P values <0.05 were considered statistically significant.

Results

The results of all parameters studied in different groups are shown in table (1). The significant correlations detected in CRF and HD groups between the studied parameters are illustrated in table (2). Stepwise multiple regression analysis revealed that hs CRP is a significant independent determinant of serum fetuin A ($B = -27.946$, $p = 0.000$) table (3) and that hs CRP as a marker of inflammation together with t-PA as a marker for fibrinolysis, are significant independent determinant of serum fetuin A ($B = -31.702$, $p = 0.000$), ($B = 1366.314$, $p = 0.000$) respectively} table (4).

Table (1): Results of the parameters studied in all groups

	Control group	Group A (CRD)	Group B (HD)
Creatinine (mg/dl)	0.80 \pm 0.30	4.81 \pm 0.068 ^a	9.21 \pm 1.51 ^{ab}
Fetuin-A (μ g/ml)	632.71 \pm 69.53	516.00 \pm 64.45 ^a	433.67 \pm 67.03 ^{ab}
hs CRP (mg/l)	2.05 \pm 1.45	7.28 \pm 1.63 ^a	11.25 \pm 1.54 ^{ab}
IL-6 (pg/ml)	0.55 \pm 0.20	2.64 \pm 0.89 ^a	5.72 \pm 1.81 ^{ab}
TM (ng/ml)	55.40 \pm 14.41	201.20 \pm 27.93 ^a	323.67 \pm 44.62 ^{ab}
vWF (%)	53.67 \pm 6.27	92.23 \pm 11.20 ^a	130.92 \pm 16.21 ^{ab}
t-PA (ng/ml)	0.243 \pm 0.047	0.605 \pm 0.082 ^a	1.59 \pm 0.33 ^{ab}
PAI-1 (ng/ml)	6.97 \pm 1.83	13.83 \pm 2.86 ^a	24.05 \pm 5.68 ^{ab}
D-dimer (ng/ml)	262.67 \pm 49.64	427.33 \pm 52.30 ^a	742.00 \pm 109.95 ^{ab}

a: Significant difference from control group

b: Significant difference from group A (CRD group)

Significant difference: $p < 0.05$

Table (2): Significant correlations obtained in groups A&B between parameters studied

Correlations	Group A (CRD) Correlation coefficients	Group B (HD) Correlation coefficients
Fetuin-A vs creatinine	- 0.924**	- 0.941**
Fetuin-A vs hs-CRP	-0.980**	-0.948**
Fetuin-A vs IL-6	- 0.953**	-0.944**
Fetuin-A vs TM	-0.886**	-0.976**
Fetuin-A vs vWF	- 0.875**	- 0.842**
Fetuin-A vs t-PA	- 0.835**	-0.750**
Fetuin-A vs PAI-1	- 0.935**	- 0.938**
Fetuin-A vs D-dimer	- 0.849**	-0.874**
t-PA vs IL-6	0.767**	0.729**
t-PA vs hs CRP	0.823**	0.756**
PAI-1 vs IL-6	0.941**	0.962**
PAI-1 vs hs CRP	0.934**	0.933**

** Correlation is significant at the 0.01 level

Table (3): Stepwise multiple regression analysis of serum fetuin A and hs CRP in whole patients groups

	B	T	P
Constant	733.739	35.064	0.000*
hs CRP	-27.946	-12.817	0.000*

R=0.924, $R^2 = 0.854$, SE= 29.90

* Statistically significant at the level of $p < 0.01$

Table (4): Stepwise multiple regression analysis of serum fetuin A and hs CRP with T-PA in whole patients groups

	B	T	P
Constant	692.124	35.574	0.000*
hs CRP	-31.702	-16.185	0.000*
t-PA	1366.314	4.143	0.000*

R=0.954, $R^2 = 0.911$, SE= 23.81

* Statistically significant at the level of $p < 0.01$

Discussion and Conclusion

ED is common in patients with CRD and in patients on renal replacement therapy such as HD [14]. Progression of CRD is associated with both decreased endothelial function and increased prevalence of atherosclerosis, and vascular media calcification. All of which have been associated with mortality. ED precedes clinically detectable vascular disease [15-17]. It has been recognized for over 150 years that abnormalities in blood flow, vessel wall and blood components may contribute towards thrombosis (Virchow's triad) [18]. This simplified view is now modified by the recognition that the process of thrombus formation (thrombogenesis) requires complex interactions involving injury to the vascular endothelium, platelet adherence, aggregation and release, and clotting factor activation; this process eventually leads to thrombin generation and fibrin formation [19]. Under physiological conditions, the vascular endothelium produces many substances which are closely associated with hemostasis, fibrinolysis, synthesis of growth factors, and the regulation of vessel tone and permeability. The etiology of ED is complex and involves dysregulation of multiple pathways [20].

In the present study, serum fetuin-A was measured in CRD and HD patients in a trial to clarify its potential contribution to ED and haemostatic alteration frequently encountered in these patients. The results obtained revealed significant reduction in Fetuin-A levels in CRD and HD patients compared to controls. These findings are in accordance with previous studies [21-24]. On the other hand, Ix et al., [25] reported that serum fetuin-A concentration was not reduced in 970 patients with mild CRD. Caglar et al., [13] found that serum fetuin-A concentrations were decreased in all stages of CRD except stage 1. On the basis of these observations, it could be suggested that serum fetuin-A levels decline during

the course of progression of CRD. This suggestion could be supported by the highly significant inverse correlation detected between serum fetuin-A and creatinine in CRD and HD patients included in the present work. The results also revealed significant decrease in serum fetuin-A levels in HD group in comparison to CRD group. Similar finding was reported by Ketteler et al [21] and Balon et al., [26]. A significant decrease of fetuin-A levels after a single HD session was also detected by Ciaccio et al., [27] and Errakonda et al., [28]. The results of the present study are in accordance with previous studies (Weinhol et al., [29] and Chang et al., [30], showing that CRD and HD patients had higher levels of inflammatory markers, as evidenced by the significant increase in hs CRP and IL-6 levels, in comparison to controls. Additionally, HD patients had significantly higher CRP levels compared to CRD patients. Chronic low-grade inflammation is a common feature of CKD. The causes of the highly prevalent state of inflammation in CKD are multiple and include factors such as volume overload, comorbidity, intercurrent clinical events, the dialysis procedure *per se* as well as genetic factors [31, 26]. Chronic inflammation in CKD patients may contribute to down regulation of fetuin-A serum levels [32, 33, 24]. Our results revealed a strong inverse correlation between serum fetuin-A and each of hs CRP and IL-6. In addition, regression analysis revealed that hs CRP is an independent determinant of serum fetuin-A level. These findings support the hypothesis of inflammation-dependent down-regulation of fetuin-A expression and highlight the close relationship between inflammation and vascular calcification in CRD.

To evaluate the potential role of fetuin-A in endothelial cell activation and damage which could induce thrombosis, several measures of endothelial function and fibrinolytic parameters were assessed in

the current study. The traditional biomarkers of ED namely, TM and vWF were evaluated. They were significantly increased in CRD and HD patients compared to controls. The increase was also significant when HD patients were compared to CRD patients. This increase reflects the endothelial cell injury and denotes the presence of ED in CRD patients which was more pronounced in HD patients. Although ED in CRD and HD patients has been reported by many authors [7, 34, 35, 36], the precise mechanism that induces it is not clear. There are many postulations among which the accumulation of certain uremic factors [37], systemic inflammation which is closely associated with augmented oxidative stress [38], decreased oxygen supply to endothelial cells as a result of anemia [39], hypertension and shear stress [40], and the massive release of cytokines during dialysis [38]. Recent studies have demonstrated a possible role of fetuin-A in the pathogenesis of ED in CRD [41-43]. The present study demonstrated that the circulating biomarkers of ED (TM and vWF) showed a progressive and significant increase, in relation to the decrease of fetuin-A in CRD and HD patients. These findings are in accordance with those reported by previous studies [13,43] and support the hypothesized that low serum fetuin-A with subsequent vascular calcification could be one of the contributing factors for the development of ED in CKD and HD patients.

vWF is synthesized by, and stored in, endothelial cells. When released, it appears to mediate platelet adhesion and aggregation. The close association between vWF and the processes of thrombogenesis suggests that high vWF levels may be a useful indirect indicator of thrombosis [44] and also suggests that high vWF levels could contribute to thrombotic episodes in CRD and HD patients. The significant inverse correlation between fetuin-A and vWF clarifies the possible role played by fetuin-A in inducing thrombosis in CRD and HD patients.

The results of this study revealed that CRD and HD patients had significantly increased fibrinolytic parameters, t-PA, PAI-1 and D-dimer, compared to controls. HD patients had significantly higher values of the previously mentioned parameters in comparison to CRD patients. Gray et al., [45] and Bono,[46] stated that in patients with ischemic heart disease and diabetes respectively there is an approximately linear relation between t-PA and PAI-1 plasma concentrations; the equilibrium of the reaction is such that high t-PA and higher PAI-1 antigen concentrations are associated with low t-PA activity. The significantly increased t-PA, PAI-1 and D-dimer levels in our study population are consistent with a procoagulant state usually associated with the underlying uremia, ED and proinflammatory

changes. This finding was clarified by the significant inverse correlation between fetuin-A and each of t-PA, PAI-1 and D-dimer, in addition to the significant direct correlation between each of t-PA and PAI-1, and the inflammatory markers (hs CRP and IL-6) in CKD and HD patients studied. Similar findings have been reported by previous authors and they indicated that the increase in t-PA and PAI-1 reflected a mediated or facilitated endothelial cell injury as supported by certain clinical and experimental data [43, 47, 48]. An alternative explanation was that PAI-1 is an acute-phase protein that can rise in response to several stimuli, including cytokines such as IL-6. It has been speculated that the development of atherothrombotic events in CRD patients is due, at least in part, to an impaired fibrinolysis [49]. Regression analysis data revealed that hs CRP, as a marker of inflammation, together with t-PA, as a marker for fibrinolysis, are significant independent determinant of serum fetuin A which prove the impact of inflammation and fibrinolysis on serum fetuin A in patients with CRD.

In conclusion, the results of the present study seem to indicate that in CKD and HD patients inflammatory processes are increased and linked to low fetuin-A and vascular calcification which represents a novel risk factor for the development of ED. The interplay of these phenomena could be responsible for the development and progression of accelerated thrombogenesis that is peculiar to renal patients. Therefore, the use of therapeutic agents, such as sevelamer, which decrease inflammation and increase levels of the fetuin-A could be tried in CRD and HD patients to improve the endothelial function and minimize the thrombotic complications.

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11/1/2010