

## ORIGINAL ARTICLES

### Is The Circulating Calcification Inhibitor, Fetuin-A Associated With Vascular Stiffness And Calcification In Children On Dialysis?

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#### ABSTRACT

**Background:** Vascular calcification occurs in the majority of patients with chronic kidney disease, but a subset of patients do not develop calcification despite exposure to a similar uraemic environment. The physiological inhibitor of calcification, fetuin - A, may play a role in preventing the development and progression of ectopic calcification, but there are scarce and conflicting data from clinical studies. **Methods:** We measured serum fetuin-A, in 30 end stage renal failure (ESRF) children on haemodialysis and compared the results with those of 30 normal children of same age and sex as controls. We also studied its association with vascular calcification and biochemical measures. **Results:** Serum fetuin A level was significantly higher in children on dialysis than in controls ( $352 \pm 46.3$  Vs  $300.5 \pm 9.9$   $\mu\text{g/ml}$ ) ( $P < .05$ ). There is a significant increase of blood levels of fetuin- A in children on dialysis without vascular calcification as compared to those with calcification ( $385.4 \pm 46.6$  Vs  $311 \pm 7.6$ ) ( $P < .0001$ ). No significant correlation was found between serum fetuin- A and blood levels of parathormone, phosphorus, calcium, and calcium – phosphorus products ( $r = 0.16, 0.15, 0.07$  and  $0.02$  respectively). **Conclusion:** Repeated measurement of serum fetuin - A levels may add significant prognostic information to patients on hemodialysis. Moreover this negative acute phase reactant may represent a potential therapeutic target in an attempt to decrease cardiovascular mortality in ESRF children on hemodialysis.

**Key words:**

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#### Introduction

Cardiovascular disease is an important predictor of mortality in patients with end-stage renal disease (ESRD) and accounts for almost 50% of deaths in these patients (Ossareh 2011). Structural and functional abnormalities and calcification in the large vessels began as early as the first decade of life in patients with chronic kidney diseases (CKD) (Shroff *et al.*, 2007). Arterial calcification and especially coronary artery calcification is known as a risk factor for cardiovascular disease in these patients, and cross-sectional and longitudinal studies on ESRD patients have shown that arterial calcifications are associated with cardiovascular morbidity and are an independent predictor of cardiovascular mortality (London *et al.*, 2003 and Blacher *et al.*, 2001). Arterial medial calcification has been shown as a strong prognostic marker of cardiovascular mortality in hemodialysis patients, independent of classical atherogenic factors (London *et al.*, 2003). The main question is what causes and drives this early and extensive vascular calcification in children with chronic kidney disease (CKD), and what are the main strategies to prevent or possibly reverse it?

In fact, all extracellular fluid contains Ca and PO<sub>4</sub> in concentrations exceeding their solubility product for spontaneous precipitation, suggesting that under normal conditions inhibitors of calcification prevent the development and progression of vascular calcification (Westenfeld *et al.*, 2007).

A number of local and systemic calcification inhibitors including fetuin-A, matrix Gla protein and osteoprotegerin have been identified in recent years (Ketteler *et al.*, 2005).

Fetuin-A is a major systemic inhibitor of calcification, accounting for approximately 50% of the precipitation inhibitory capacity of serum (Ketteler *et al.*, 2005). Fetuin - A is a hepatocyte-derived serum protein (molecular weight, ~60 kD). Serum concentrations are relatively high with levels between 0.5 and 1.0

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g/L in average populations. Fetuin-A is an inhibitor of calcification, limiting hydroxyapatite crystal formation (DelleGrottaglie *et al.*, 2006). In vitro, fetuin-A is a highly potent inhibitor of hydroxyapatite formation and reduces crystal formation in solutions containing calcium and phosphate (Heiss *et al.*, 2003). In vivo, depending on their genetic background, fetuin-A-deficient (Fetuin- A<sup>-/-</sup>) mice develop severe soft tissue calcifications either spontaneously or following challenges with vitamin D, or by feeding a mineral-rich diet (Schafer *et al.*, 2003).

#### *Aim of the work:*

In the present study, we aimed to investigate the relationship between fetuin-A, and coronary artery calcification in children with End Stage Renal Disease (ESRD) under hemodialysis.

#### *Patients and methods:*

##### *Study design:*

Thirty consecutive children aged 3- 16 years (13) girls and (17) boys , who had been on haemodialysis for  $\geq 3$  months due to end stage renal disease (ESRD) were recruited in the study. They were on haemodialysis at children Hospital, Cairo University. The duration of dialysis ranged from 3 to 84 months. The primary causes of renal failure were : pyelonephritis in eight patients, glomerulonephritis in four, interstitial nephritis in eight, urolithiasis in three, bilateral atrophic kidney in four, one with hemolytic uremic syndrome, one with congenital renal hypoplasia, and one with renal polycystic disease.

Patients were compared with 30 healthy children as controls. Controls were age and gender matched with the dialysis cohort and confirmed to have no known medical illnesses, family history of heart disease or active infections at the time of the study.

All patients underwent clinical examination including: Mean $\pm$  S.D, Weight (22.68 $\pm$ 7.2 kg), height (113.17 $\pm$ 15.03cm), systolic blood pressure (112.67 $\pm$  12.85mmhg) and diastolic blood pressure (74 $\pm$  8.94 mmhg).

Patients were examined by echocardiography using 2D m mode modality, calcification was found in 4 patients, Left ventricular hypertrophy was found in 8 patients, mitral valve regurge in 4 patients, Tricuspid regurge in 2 patients , 15 patients were normal .

##### *Methods:*

About 5 ml of fasting, over night, venous blood sample was taken from each child participating in this study and divided into aliquots:

- The 1<sup>st</sup> aliquot about 1ml of venous blood was added into a tube containing EDTA for hemoglobin and haematocrite value determination using Coulter Counter T 890 (Coulter Counter, Harpenden, UK).
- The 2<sup>nd</sup> aliquot about 4 ml of venous blood was left to clot and the serum was separated by centrifugation and stored at -20°C for determination of urea, creatinine, alanine aminotransferase, alkaline phosphatase, sodium, potassium, calcium, phosphorous, parathormone (PTH) and fetuin-A.

The determination of serum urea, creatinine, alanine aminotransferase, alkaline phosphatase, sodium, potassium, calcium and phosphorous were carried out on Olympus AU 4000 analyzer (Europe GmbH, Wendenstraße, Hamburg, Germany). The kits were supplied by Olympus Life and Material Science (Hamburg, Germany).

The determination of PTH was carried out using ELISA method (Woodhouse *et al.*, 1997). The kit was supplied from Diagnostic Systems Laboratories, Inc.(445 Medical Center Blvd Webster Texas).

The determination of serum fetuin-A was carried out by immunoturbidimetric method (Müssig *et al.*, 2009), on RA 50 (Bayer's Diagnostic, USA) and the kit was supplied by Bio Vendor Laboratory Medicine (Bio Vendor GmbH, Neuenheimer Feld, Heidelberg, Germany).

##### *Results:*

The results of this study were illustrated in tables; 1, 2 and 3.

Table (1) showed the demographic, clinical, anthropometric and biochemical characteristics of ESRD children undergoing dialysis and normal controls, it revealed:

- A significant increase in blood levels of fetuin-A in ESRD children undergoing dialysis when compared to normal controls ( 352 $\pm$  46.3 Vs. 300.5  $\pm$  9.9 ) (P< .05).

- A highly significant increase in blood levels of PTH in ESRD children undergoing dialysis when compared to normal controls ( $416.5 \pm 243$  Vs.  $30.8 \pm 8$ ) ( $P < .0001$ ).
- An insignificant decrease in blood levels of calcium in ESRD children undergoing dialysis when compared to normal controls ( $8.5 \pm 0.7$  Vs.  $8.6 \pm 2.3$ ).
- An insignificant decrease in blood levels of phosphorus in ESRD children undergoing dialysis when compared to normal controls ( $4.3 \pm 1.1$  Vs.  $4.7 \pm 0.7$ ).

Table (2): showed the Mean  $\pm$  S.D of serum levels of fetuin-A, PTH, phosphorus and calcium among ESRD children undergoing dialysis with and without vascular calcification diagnosed by ECHO, it revealed:

- A significant increase of blood levels of fetuin-A in ESRD children undergoing dialysis without vascular calcification when compared to those with calcification ( $385.4 + 46.6$  Vs.  $311 + 7.6$ ) ( $P < .0001$ ).
- A significant decrease of blood levels of PTH in ESRD children undergoing dialysis with vascular calcification when compared to those without calcification ( $365.3 + 179$  Vs.  $100 + 15.8$ ) ( $P < .01$ ).
- An insignificant increase of blood levels of phosphorus in ESRD children undergoing dialysis with vascular calcification when compared to those without calcification ( $4.35 + 1.2$  Vs.  $4.24 + 1.1$ ) ( $P > 0.05$ ).
- An insignificant increase of blood levels of calcium in ESRD children undergoing dialysis with vascular calcification when compared to those without calcification ( $8.51 + 0.8$  Vs.  $8.5 + 0.5$ ) ( $P > 0.05$ ).

Table (3): showed the correlation coefficient between blood levels of fetuin-A and parathormone (PTH), phosphorus, calcium and calcium - phosphorus products.

- A negative insignificant correlation between blood levels of fetuin-A and PTH, phosphorus, calcium and calcium phosphorus products ( $r = -0.16, -0.15, -0.07$  and  $-0.02$  respectively).

**Table 1:** Demographic, clinical, anthropometric and biochemical characteristics of patients (N=30) and controls (N=30).

	Mean	S.D	Significance
Duration of dialysis (months)			
Cases	48.2	22.5	
Controls	-----	-----	-----
Fetuin A level(mg/dl)			
Cases	352	46.3	<.05
Controls	300.5	9.9	
Age(years)			
Cases	10.6	3.3	<.05
Controls	8.4	4	
Weight(kg)			
Cases	22.7	7.2	<.0001
Controls	17.7	8.3	
Height(cm)			
Cases	113.2	15	<.0001
Controls	98.6	16.4	
Systolic blood pressure(mmHg)			
Cases	112.7	12.8	<.0005
Controls	93.9	3.5	
Diastolic blood pressure(mmHg)			
Cases	74	8.9	N.S
Controls	76.9	4.8	
Creatinine before dialysis (mg/dl)			
Cases	8.5	2.6	<.0005
Controls	0.5	0.14	
Creatinine after dialysis(mg/dl)			
Cases	3.2	2.5	<.005
Controls	0.5	0.14	
Urea before dialysis(mg/dl)			
Cases	66.9	14.7	<.0005
Controls	12.7	1.9	
Urea after dialysis(mg/dl)			
Cases	14.7	8.6	N.S
Controls	12.6	1.9	
Sodium in blood (mg/dl)			
Cases	136.2	5.7	<.05
Controls	138.6	2.1	
Potassium in blood (mg/dl )			
Cases	5.2	1.1	<.0005
Controls	4.2	0.4	
Calcium in blood (mmol/L)			
Cases	8.5	0.7	N.S
Controls	8.6	2.3	
Phosphorus in blood (mmol/L)			
Cases	4.3	1.1	N.S
Controls	4.7	0.7	

ALT in blood(mg/dl)			
Cases	25.8	5.9	<.002
Controls	18.1	3.7	
Alkaline phosphatase in blood(mg/dl)			
Cases	404.1	145.5	<.001
Controls	239.2	71.7	
PTH(pg/ml)			
Cases	416.5	234	<.0001
Controls	30.8	8	
Hematocrite(%)			
Cases	29.8	5.8	<.0005
Controls	38.9	1.6	
Hemoglobin(g/dl)			
Cases	9.9	1.7	<.0005
Controls	13.1	0.7	

**Table 2:** Mean  $\pm$  S.D of blood levels of fetuin-A, PTH, Phosphrus and Calcium among ESRD children undergoing hemodialysis  $\pm$  vascular calcification diagnosed by ECHO.

	ECHO		P-value
	Patients without calcification	Patients with calcification	
Fetuin-A (mg/dl)(mean $\pm$ S.D)	385.4 $\pm$ 46.6	311 $\pm$ 7.6	<.0005
PTH (pg/ml) (mean $\pm$ S.D)	365.3 $\pm$ 179	100 $\pm$ 15.8	<.01
Phosphorus (mmol/L)(mean $\pm$ S.D)	4.24 $\pm$ 1.1	4.35 $\pm$ 1.2	NS
Calcium (mmolL)(mean $\pm$ S.D)	8.5 $\pm$ 0.5	8.51 $\pm$ 0.8	NS

**Table 3:** Correlation coefficient between blood levels of Fetuin-A, PTH, phosphorus, calcium - phosphorus products.

	PTH	Phosphorus	Calcium	Calcium-phosphorus products
Fetuin-A level				
r	-0.16	-0.15	-0.07	-0.02
P	N.S	N.S	N.S.	N.S

### Discussion:

In this study, vascular changes were observed in as many as 40% of ESRD children undergoing dialysis. Such changes with renal failure were previously reported to occur in childhood (London *et al.*, 2003).

In recent years a number of local and systemic calcification inhibitors, including fetuin-A, matrix Gla protein, and osteoprotegerin, have been identified. Deficiency and/or dysregulation of such factors may contribute to morbidity and even mortality through increasing extra-osseous calcifications such as vascular calcifications which occur with high prevalence in patients with ESRD (Turkmen *et al.*, 2011).

Although several studies have been reported that adults on dialysis have significantly lower serum levels of fetuin-A than controls (Ketteler *et al.*, 2003 and Wang *et al.*, 2005), we found higher serum levels of fetuin- A in ESRD children undergoing dialysis as compared to healthy age matched controls. It is noteworthy to mention that, children with evidence of calcification on ECHO had lowered serum levels of fetuin-A when compared to those without calcifications, but even this group had higher serum levels than those of the controls. Really the role of serum fetuin-A levels in vascular calcification may be far more complex than previously thought (Kirkpantur *et al.*, 2009). The elevated fetuin-A level seems to protect children with ESRD from developing vascular calcifications (Turkmen *et al.*, 2011). This protective mechanism allows an up-regulation of fetuin- A in the early stages of CKD and dialysis, and only severe or prolonged exposure to pre-inflammatory and / or pro-calcific environment eventually leads to low fetuin A levels due to reduced production and/or increased consumption (Shroff RC *et al.*, 2008). Moreover, the basal fetuin- A level significantly and inversely correlated with basal calcium phosphorus product. It is conceivable that increased calcium phosphorus product may influence fetuin-A uptake and subsequent degradation (Pertosa 2009).

The differences in serum levels of fetuin A may be consistent with a different pathogenetic role of it in different stages of chronic renal disease (Wigger *et al.*, 2009 and Mehrotra *et al.*, 2005). Additionally, it may be difficult to assess the relationship between vascular calcification and serum fetuin-A levels in a cross-sectional study as vascular calcification is a slowly progressive process and serum fetuin-A levels may fluctuate, possibly dependent on flares of inflammation (Caliskan *et al.*, 2010).

In addition, the genetic polymorphisms of fetuin A may indeed play a role in an individual patient's susceptibility to calcify, possibly by modulating the magnitude of changes in fetuin-A production in response to a pro-inflammatory or pro-calcific environment (Stenvinkel *et al.*, 2005). So, patients with calcification may have genetically lower fetuin-A levels that predispose them to calcification (Cozzolino *et al.*, 2007).

Most cytokines and growth factors involved in the pathogenesis of atherosclerosis relay for their cellular effects on either receptor associated or cytoplasmic tyrosine kinase (Heldin 2001). Fetuin-A has a high degree of structural similarity to a rat peptide tyrosine kinase inhibitor (Haasemanne *et al.*, 1991) and thus fetuin-A was shown to modulate, at least in vitro, effects of different growth factors interacting with a tyrosine kinase receptor

(Heldin 2001). Thus this class of enzymes has been suggested as a potential therapeutic target to prevent the progression of vascular damage in atherosclerosis (Levitzki 2005), therefore, it is conceivable that fetuin A through its effects on cell signaling, may modulate the action of several atherogenic mediators, acting as a circulating inhibitor of atherosclerotic plaque formation and/or progression (Singh & Ramji 2006 and Szweras *et al.*, 2002).

The association between reduced fetuin-A levels and cardiovascular mortality (Stenvinkel *et al.*, 2005 and Hermans *et al.*, 2007) in many large studies suggests that fetuin-A is likely to have a causal effect on vascular calcification. Moreover, low circulating fetuin-A levels are associated with high serum PO<sub>4</sub> levels even in the general population and have been associated with valvular calcification in patients with normal renal function (Ix *et al.*, 2006). As the development of such vascular changes as early as childhood is related to worse prognosis later in life, we need to consider whether determination of fetuin-A level should become a routine test in children with renal failure (Westenfeld *et al.*, 2009)

In this study, while the blood levels of PTH, were higher in children with ESRD and undergoing hemodialysis than in the control group, the parameters of the calcium-phosphorus metabolism balance did not differ greatly between patients and normal controls. Our results run parallel with Goodman *et al.* (2000), who found an insignificant trend for higher PTH levels in patients without coronary arteries calcification. These results are supported by the results of Poyrazoğlu *et al.*, (2007), who found a significant negative correlation between carotid calcification and PTH levels.

The role of parathyroid hormone (PTH) in vascular calcification is not yet clear. Chertow and colleagues (2002), showed that lowered serum levels of PTH are associated with more extensive calcification especially in calcium-treated subjects and they suggested that PTH may have a protective effect against vascular calcification, and the severity of vascular calcification may increase in conditions of adynamic bone disease and low PTH. Generally, PTH and PTH-related protein are assumed to induce bone demineralization on one hand and inhibit vascular calcification on the other hand, in concert with other inhibitors of mineralization of VSMCs such as fetuin-A, matrix Gla protein, and vitamin K.

Abnormalities in mineral metabolism have been accused for the development of vascular calcification in patients with CKD and ESRD. Increased phosphate level due to decreased phosphate excretion is generally observed in advanced CKD, and together with increased calcium and calcium-phosphate product, has been attributed to development of vascular calcification in these patients (Ix *et al.*, 2007). This is in accordance with our results as increased blood levels of both calcium and phosphorus were higher in ESRD children with calcification compared to those without.

Moreover, hyper-phosphataemia may play another major role in initiating changes in vessel wall cells that subsequently lead to the development of calcifications. Another trigger required for initiation of the mineralization process is the separation of both living and apoptotic cells and the matrix vesicles from cells that perform the function of mineralization centers. One such factor stimulating freeing of the matrix vesicles is the intracytoplasmic phosphate level (Reynold's *et al.*, 2004).

It is noteworthy to mention that, in uremia, the typical metabolic disturbances including lowered serum levels of fetuin-A and increased serum levels of phosphorus, calcium×phosphate product, parathyroid hormone, and an increased calcium intake, are independent predictors of cardiovascular morbidity and mortality in this population (Ganesh *et al.*, 2001).

#### Conclusion:

According to our data repeated, serum fetuin-A evaluation may add significant prognostic information in vascular calcification in ESRD children undergoing dialysis. We show that high serum levels of fetuin-A correlates with decreased vascular calcification, suggesting that modulation of this negative acute phase reactant may represent a potential therapeutic target in the attempt to decrease the cardiovascular risk of haemodialysis patients.

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