Serum Fetuin-A in Egyptian Pediatrics with Chronic End-stage Renal Diseases: A Correlation to Vascular Inflammatory Biomarkers

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Abstract: Background: The incidence of vascular calcification is known to be elevated in haemodialysis patients. A close relationship does also exist between inflammation and vascular calcification. **Aims:** To understand role of inflammation; we investigated the serum concentrations of some inflammatory markers in children with end stage renal diseases, and compared those patients either undergoing haemodialysis or not with their control counterparts. The potential relationship between serum fetuin-A and the inflammatory markers in the studied patients was also unraveled. **Methodology:** In this prospective non-randomized study, eighty chronic kidney failure pediatric Egyptian patients were enrolled. Group I included 40 of them who were managed without haemodialysis, while group II included 40 on regular haemodialysis in the Center of Pediatric Nephrology and Transplantation (CPNT), Cairo University Children's Hospital, Egypt; both groups were compared with a control group of 40 subjects with normal renal function. Serum fetuin-A, IL-1b, IL-23, TNF-α, circulating soluble intercellular (sICAM-1) and vascular cellular (sVCAM-1) adhesion molecules were measured by ELISA. **Results:** Comparing the hemodialized patients in group II with the non-hemodialized in group I**,** IL-1b was significantly lower in group II (p=0.023), whereas, other parameters showed insignificant decrease (sICAM-1, p=0.223, sVCAM-1, p = 0.209, TNF-α, p =0.059, IL-23, p =0.339, Fetuin-A, p =0.236). All inflammatory markers were significantly higher in both patient groups I and II, compared to their control, except for fetuin-A which showed a significant decrease. Statistically significant negative linear correlations were found between fetuin-A and all inflammatory markers among all studied patient groups and within each group separately. **Conclusion**: Our data suggest that the immune cells in hemodialized children are in an activated state which explains the induced alterations in the assessed immunological markers. An important negative associations between serum fetuin-A and serum inflammatory markers concentrations were also demonstrated.
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1. Introduction

It is estimated that 1 in 9 individuals in the United States have some manifestations of chronic kidney disease (CKD), ranging from proteinuria with normal renal clearance/function to advanced renal failure requiring renal replacement therapy in the form of dialysis or transplantation, commonly called end-stage renal disease (ESRD) (NKF, 2002).

In the general pediatric population, the incidence of annual death due to cardiac disease is less than 3%. Over the last decade cardiovascular diseases (CVDs) have remained the second most common cause of death in children on chronic dialysis or after transplantation, accounting for approximately 20–25% of all deaths (Parekh *et al.,* 2002). This is likely from (1) the extremely high prevalence of atherosclerosis, heart failure, and left ventricular failure in hemodialysis patients, observed in 40% to 74% of incident dialysis patients and (2) a high case mortality rate after an acute myocardial infarct or of heart failure (Sarnak *et al.,* 2003, Sharon *et al.,*2004).

The incidence of vascular calcification (VC) is elevated in haemodialysis (HD) patients (Cozzolino *et al.,*2006). Moreover, VC was identified as important predictor of mortality in ESRD patients on maintenance dialysis (Goodman *et al.,*2000; London *et al.,*2003; Wang *et al.,*2003a). Raggi *et al.* (2007) reported that up to 60% of patients beginning dialysis therapy and 80–85% of patients presently on HD have coronary artery calcification.

Inflammation is playing an important role in the development of VC in addition to other well-known risk factors as advanced age, duration of dialysis treatment, diabetes, high serum phosphate levels and high dosing of calcium-containing phosphate binders (Pecovnik-Balon, 2005). Proinflammatory cytokines have been shown to

enhance *in vitro* calcification of vascular cells, suggesting a close relationship between inflammation and calcification (Tintut *et al.,* 2000).

Atherosclerosis can be described as a chronic inflammatory process. It is known that several inflammatory and immunologic factors contribute significantly to atherogenesis (Ross, 1999). Furthermore, several reports suggest that the serum levels of soluble adhesion molecules may be beneficial for providing information on potential atherosclerotic lesions (Blann and McCollum, 1994; Nakai *et al.,*1995; Ross, 1999).

During the inflammatory reaction, anti-inflammatory cytokines are also produced and tend to modulate this inflammatory reaction. Fetuin-A (α2-Heremans Schmid glycoprotein; AHSG), with a molecular mass of a 59 kDa, is a predominantly liver-derived glycoprotein, and has been identified as a circulating inhibitor of calcification (Schafer *et al.,*2003). It is considered an anti-inflammatory mediator that participates in macrophage deactivation (Wang *et al.,*1998). It is also recognized that fetuin-A can actively regulate the cell-mediated process of osteogenesis in the vessel wall, inhibits mineralization in a concentration-dependent manner, and enhances the phagocytosis of apoptotic bodies by vascular smooth muscle cells (Reynolds *et al.,*2005). Moreover, fetuin-A is an antagonist of bone morphogenetic protein-2, the promoter of VC in vascular cells (Szweras *et al.,*2002).

Some studies have demonstrated an association between serum fetuin-A levels and all-cause mortality of dialysis patients (Stenvinkel *et al.,*2005; Wang *et al.,*2005). Ketteler and co-workers. (2003) reported an inverse relationship between serum fetuin-A and C-reactive protein (CRP). Serum fetuin-A showed also important associations with atherosclerosis, malnutrition; and cardiovascular events in peritoneal dialysis patients (Wang *et al.,*2005).

To assess the extent of inflammation, we investigated the serum concentrations of IL-1b, IL-23, TNF-α, circulating soluble intercellular (sICAM-1) and vascular cellular (sVCAM-1) adhesion molecules in children with ESRD, in patients managed with HD and those without HD, compared with healthy control subjects. We also aim to unravel the relationship between serum fetuin-A and the investigated inflammatory markers in those patients.

2. Subjects and methods

Patients.

In this prospective non-randomized study, we enrolled 80 ESRD. Group I included 40 pediatric ESRD patients who were managed without HD (20 girls and 20 boys, age range 5-13 years (9.7± 2.4 years). On the other hand, group II was composed of 40 pediatric ESRD patients (20 girls and 20 boys, age range 4-13 years (10.7±2.78 years) who were on regular HD in the Center of Pediatric Nephrology and Transplantation (CPNT), Cairo University Children's Hospital. The hemodialysis performed for group II was on 3-hour sessions of dialysis, three times weekly on bicarbonate dialysis with a polysulfone F-40 membrane. The range of HD duration for group II was 1-6 years, (4.01 ± 1.19).

The study was conducted through a 3 months' duration (from 1/3/2012 till 31/5/2012). *The inclusion criteria* for this study were: pediatric age (≤ 13 years) when diagnosed with ESRD, glomerular filtrations rate < 15 ml/min/1.73 m^2 and minimum dialysis duration of one year (for group II). For group I, participation was allowed if the creatinine clearance was below 59 ml/min (measured directly by clearance technique). All of the included patients were clinically stable, and free of active infection during the study period.

The exclusion criteria included presence of diabetes mellitus, autoimmune disease, and acute kidney injury or with unsatisfactory vascular access affecting dialysis adequacy or any other known condition that would alter cytokine levels. Moreover, none of our patients had received antibiotics, anti-inflammatory or corticosteroid medications during the study period.

Forty volunteers (age and sex matched) had normal renal function (serum creatinine levels less than 1.4 mg/dl), were involved in our study as a healthy control group.

The study protocol was approved by the local ethics committee, and informed written consent was obtained from the parents of the patients and volunteers before entering the study.

Hemodialysis.

Hemodialysis machines with volumetric control (Fresenius Medical Care 4008B and 4008S, Homburg, Germany) were used. The standard dialysis bath consisted of sodium, 140 mEq/L; potassium, 2 mEq/L; calcium, 3 mEq/L; and bicarbonate, 35 mEq/L. The ultra filtration rate was programmed to reach the patient's optimal dry weight defined as the post-dialysis body weight below which the patients developed symptomatic hypotension or muscle cramps in the absence of edema. Heparin was used for anticoagulation. Total weekly urea clearance (Kt/V) and creatinine clearance (CCr) were used to measure dialysis adequacy as described previously by Wang *et al.* (2003 b)

Biochemical analysis.

Following an overnight fasting not less than 12 hrs, morning (8:00-9:00 AM), five mls venous blood samples were collected for measurement of serum parameters.

PTH was determined by immunoradiometric assay using Elecsys 2010 autoanalyzer system (Roche Diagnostics, Basel, Switzerland). Serological determination of serum fetuin-A was done using a human fetuin-A enzyme linked immunosorbent assay (ELISA) kit (BioVendor GmbH, Heidelberg, Germany). sICAM-1, sVCAM-1, TNF-α., IL-1b and IL-23 were also analyzed by an ELISA kit (Thermo Fisher Scientific Inc, USA). All samples were analyzed in duplicate.

Serum samples for measurement of other parameters were analyzed using Synchron cx5 autoanalyzer (Beckman, USA). Serum C-reactive protein (CRP) levels at the time of investigation were retrospectively obtained from patient records.

Statistical analysis.

All data were expressed as mean ± SD. All analyses utilized SPSS 17.0 statistical package for Windows (SPSS Inc., Chicago, IL). A one-way analysis of variance (ANOVA) was employed for comparisons of means of the different groups. A *p*-value < 0.05 was accepted as statistically significant using Bonferroni Post HOC test. Correlation analyses were done using Spearman correlation.

3. Results

The statistical comparison of HD patients (group II) with group I showed significant decreases in serum albumin, phosphate, calcium, urea, creatinine, sodium, potassium, TSH as well as the lipid profile (all at $p<0.00$). No significant differences were found in the levels of alkaline phosphatase(ALP), PTH, T3 and T4 (Table1). Furthermore, by comparing the biochemical markers between each patient group I and II with their control counterparts, significant differences ($p<0.05$) group were detected. The levels of Alb, Ca and TSH were significantly decreased in both groups of patients, while phosphates, urea, creatinine, T3, T4 and PTH were significantly elevated (Table 1).

The comparison between the concentrations of inflammatory markers between each patient group (I and II) with the control group (Table 2) using Post HOC Boferroni correction showed a significant elevation in their levels within patients with the following extents; IACAM-1 (7-8 fold), VACM-1 (7-7.5 fold), TNF-α (20.5-22.5 fold), IL-1b (43.5- 46 fold) IL-23 (20 fold); all at p =0.00. Interestingly, fetuin-A showed a significant decrease (p= 0.00) in its level in both patients groups I & II than controls (-75.7%, -78.3% , respectively).

Comparing the inflammatory markers of group II with group I (Table 2), only IL-1b level was significantly lower in group II HD patients (p=0.023), whereas, other parameters showed insignificant decrease in their level (ICAM-1, p=0.223, VCAM-1, p= 0.209, TNF-α, p=0.059, IL-23, p=0.339, Fetuin, p=0.236).

The correlational analyses of the assessed parameters are illustrated in (Table 3). It reveals significant negative linear correlations between fetuin-A and all inflammatory markers in each group separately as well as among all groups. A statistically significant negative linear correlation was detected among all ESRD patients between fetuin-A and each of ICAM-1, VCAM-1, TNF-α, IL-1b and IL-23 (r = -0.952, -0.949, -0.0906, -0.779 and -0.93, respectively, all at $p<0.01$).

4. Discussion

The lifespan of patients with ESRD is reduced, and CVDs account for a premature death in more than 50% of patients from Western Europe and North America undergoing regular dialysis (Foley *et al.,* 1998, Menon *et al.,* 2005). Actually, the risk for CVD in a 30-yr-old ESRD patient is similar to the calculated risk of a 70 to 80-yr-old subject from the non-renal population (Naschimento *et al.,* 2002).

In the current study the level of fetuin-A is found to be lower in HD patients than those without dialysis, however did not reach significance (p=0.236); findings that are in alignment with Dervisoglu *et al.* (2008). The study of Cozzolino *et al.* (2006) confirmed the lowering of serum fetuin-A levels, due to chronic inflammation in HD patients, and the significant association between reduced serum fetuin-A levels and multi-site CVC in HD patients. Cozzolino *et al.* (2007) reported also that a single HD session significantly lowers serum fetuin-A levels, possibly secondary to the inflammatory processes induced by HD therapy.

Serum fetuin-A concentrations in groups I & II were significantly lower compared to their control counterparts, results that are in harmony with Ketteler *et al.*(2003). In contrast, Dervisoglu *et al.* (2008) recorded that serum fetuin-A concentrations in HD group were not significantly lower than their control group.

The relationship between inflammation and serum fetuin-A levels in HD patients was previously investigated by Ketteler *et al.*(2003). They found that serum fetuin-A levels were negatively correlated with CRP levels, indicating that the regulation of fetuin-A is part of an acute–phase response. A negative correlation between serum fetuin-A and CRP levels was also detected in our setting, but the *P* value was 0.075 in the total patients. The retrospective nature of the CRP data could have contributed to the lack of statistical significance of our findings.

Table 1. Biochemical parameters for the 80 ESRD patients compared to their control group

	Group I (40 patients)	Group II (40 patients)	p-value (#)	Control (40 healthy subjects)	p-value (*)
Albumin (g/dl)	3.4±0.18*	3.0 ±0.12*#	0.000	4.1±0.21	0.000
Alkaline phosphatase (IU/l)	449.3±158.42*	436.6±114.98*	0.682	162.3±17.01	0.000
PO4 (mg/dl)	6.5±0.48*	4.9±0.50*#	0.000	3.8±0.25	0.000
Ca (mg/dl)	7.66 ±0.59*	6.6±0.42*#	0.000	9.2±0.25	0.000
Urea (mg/dl)	150.0±12.26*	64.8±7.83*#	0.000	20.1±3.14	0.000
Creatinine (mg/dl)	9.0±0.44*	5.3±0.34*#	0.000	0.8±0.08	0.000
Na (mmol/l)	143.6±3.96*	133.2±2.81#*	0.000	137±1.95	0.000
K (mmol/l)	5.7±0.58*	3.6±0.42*#	0.000	3.9±0.20	0.000
T_3 (ng/dl)	197.9±25.41*	202.6±26.25*	0.418	128.0±21.55	0.000
T_4 (ng/dl)	11.6±1.71*	11.8±1.67*	0.621	8.0±0.85	0.000
PTH (ng/dl)	64.4±16.54*	69.3±16.21*	0.182	4.4±0.97	0.000
TSH (ng/dl)	0.90±0.36*	0.6±0.27*#	0.000	2.8±0.84	0.000
Lipid profile HDL (mg/dl)	43.7±4.85*	38.3±4.67*#	0.000	43.8±3.68	0.000
LDL (mg/dl)	100.6±8.08*	85.5±7.85*#	0.000	93.3±10.25	0.000
Cholesterol (mg/dl)	161.8±11.58**	140.5±10.85*#	0.000	155.4±11.56	0.000
TG (mg/dl)	87.6±5.55	82.1±5.46*#	0.000	91.4±5.64	0.000

Values shown are means (± SD). n = 40 individuals per group.

(*) P-value; values are significantly different from the control group at <0.05 level; using post Hoc Bonferroni correction.

(#) P-value, values are significantly different from group I at <0.05 level; using post Hoc Bonferroni correction

Table 2. Inflammatory markers and fetuin in the different studied groups

Marker	Group I (40 patients)	Group II (40 patients)	p-value(#)	Control (40 healthy subjects)	p-value*
ICAM-1(pg/ml)	814.6±160.40*	770.2±163.03*	0.223	107.8±31.40	0.000
VCAM-1(pg/ml)	1713.9±336.63*	1619.1±332.16*	0.209	228.3±6.27	0.000
TNF-α (pg/ml)	167.0±34.52*	152.3±34.12*	0.059	7.4±1.93	0.000
IL-1b (pg/ml)	954.0±159.44*	870.2±163.07*#	0.023	20.7±4.91	0.000
IL-23 (pg/ml)	339.1±152.09*	306.1±154.12	0.339	15.7±3.19	0.000
Fetuin (µg/ml)	37.4±16.65*	33.5±15.87*	0.236	154.4±30.14	0.000

Values shown are means (± SD). n = 40 individuals per group

(*) P-value; values are significantly different from the control group at <0.05 level; using post Hoc Bonferroni correction.

(#) P-value, values are significantly different from group I at <0.05 level; using post Hoc Bonferroni correction

Table 3. Correlation between fetuin A and inflammatory markers in ESRD patients

Marker	group I		group II		All patients	
	Spearman's rho	Sig.	Spearman's rho	Sig.	Spearman's rho	Sig.
ICAM-1	-.990**	0.000	-.987**	0.000	-.952**	0.000
VCAM-1	-.989**	0.000	-.984**	0.000	-.949**	0.000
TNFα	-.982**	0.000	-.971**	0.000	-.906**	0.000
IL-1b	-.968**	0.000	-.927**	0.000	-.779**	0.000
IL-23	-.975**	0.000	-.965**	0.000	-.930**	0.000

** Correlation is significant at 0.01 levels (two tailed)

The reasons for the increased risk of inflammation in ESRD patients appear to be complex, including nondialysis- as well as dialysis-related factors. The combination of an impaired immune response coupled with persistent immune stimulation may have a role in the low-grade systemic inflammation and altered cytokine balance that characterizes the uremic state which may translate into increased risk for vascular disease (Stenvinkel and Alvestrand, 2002; Stenvinkel *et al.,* 2005).

We have detected a statistically negative significant linear correlation between fetuin-A and all inflammatory markers among all patients and also each in group separately (Table 3), whereas, in control

subjects, the correlation between serum fetuin-A and TNF-α (r = - 0.176, *P*= 0.626), and that between serum fetuin-A and IL-1b (r = -0.109, *P* = 0.763) were not statistically significant. These interesting findings support the hypothesis that inflammation itself reduces fetuin-A by down-regulation of its hepatic synthesis (Ketteler *et al.,* 2005; Moe and Chen, 2005; Cozzolino *et al.,* 2006).

Haemodialysis patients and CKD patients, not yet on dialysis, have been found to have significantly higher levels of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α. These results coincide with previous findings of Herbelin *et al.* (1991), Pereira *et al.* (1994); van Riemsdijk-van Overbeeke *et al.*(2000) and Jofre´ *et al.* (2006).

Goldstein *et al.*(2006) reported increased serum concentrations of various pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α in pediatric dialyzed patients; that were not balanced by enhanced generation of anti-inflammatory cytokines, favoring a balance toward a pro-inflammatory condition.

As the kidney is the major site for elimination of many cytokines, the delicate equilibrium of pro-inflammatory cytokines and their inhibitors is clearly dysregulated in CKD patients. The consequences of the altered immune response in uremia lead to a state of persistent inflammation which is highly prevalent among CKD patients and is linked to complications such as the development of protein-energy wasting and atherosclerotic vascular disease (Carrero *et al.,* 2008).

Premature atherosclerotic CVD is a leading cause of morbidity and mortality in patients with ESRD. Low-grade inflammation and endothelial dysfunction play pivotal roles in the initiation, progression and propagation of the atherosclerotic process (Hansson, 2005). Adhesion of circulating leukocytes to the endothelial cells and subsequent trans-endothelial migration to sites of inflammation is suggested to be an important step in the initiation and aggravation of atherosclerotic lesions (Ross, 1999). This process is predominantly mediated by cellular adhesion molecules which are expressed on the endothelial membrane in response to several inflammatory stimuli (Springer, 1990; Blankenberg *et al.,* 2003). The expression process is induced by pro-inflammatory cytokines which are present at increased levels in the uremic circulation. The expression of vascular cellular (sVCAM-1) and intercellular (sICAM-1) adhesion molecules has been demonstrated in human atherosclerotic plaques (Davies *et al.,* 1998). The study of Suliman *et al.* (2006) confirmed that high levels of soluble adhesion molecules -sICAM-1 and sVCAM-1- in incident ESRD patients starting dialysis treatment are associated with malnutrition, inflammation and CVD. They also suggested that increased levels of soluble adhesion molecules may be involved in the process of atherosclerosis and increased mortality in ESRD patients. The above studies concurred with the significant higher levels of adhesion molecules in our ESRD patients.

In patients with CKD, mediators of inflammation such as oxidation, carbonyl stress, CRP and cytokines may directly stimulate VC (Moe and Chen, 2005). Goldstein *et al.*(2006) suggested that the combination of malnutrition, inflammation, and ESRD led to cardiac calcifications in pediatric dialysis patients. Strategies to lower serum cytokine concentrations like ultra-pure dialysis with high-flux membranes, minimal catheter usage and optimal dialysis may increase serum fetuin-A concentrations, and in this fashion slow the VC process in uremic subjects (Stenvinkel *et al.,* 2005; Bommer and Jaber, 2006).

ALP is an enzyme measurable in most body fluids and usually originates from the liver or bone. In CKD patients without liver disease, ALP can be elevated in high-turnover bone disease (Regidor *et al.,* 2008). However, measuring this readily available and inexpensive biomarker has not been singled out as an individual therapeutic target of CKD (Eknoyan *et al.,*2003). This report showed significant elevation of serum ALP in ESRD patients compared to the control group, these data are supported by the study of Fahrleitner-Pammer *et al.* (2008) and Kovesdy *et al.* (2010).

An association between serum ALP level and coronary artery calcification score exists in maintenance HD patients (Shantouf *et al.,* 2009). An epidemiologic study on ~70,000 maintenance HD patients showed an increased level of ALP associated with all-cause and cardiovascular mortality. In that study, a serum ALP >120 IU/L was associated with significant death risk across almost all subgroups of HD patients (Regidor *et al.,* 2008). Moreover, Lomashvili *et al.* (2004) reported that vascular damage can induce expression of tissue nonspecific ALP, which per se hydrolyzes and inactivates inorganic pyrophosphates, a process that can enhance vascular calcification.

In healthy individuals, kidneys regulate calcium and phosphorus homeostasis through tubular re-absorption mechanism. Patients with CKD have seriously compromised homeostatic mechanism, giving rise to different adaptive changes in calcium (Ca^{2+}), phosphorus (P^{+3}), parathyroid hormone (PTH) (Mejia *et al.,* 2011). Hyperphosphatemia is a risk factor for CVD and mortality and, thus, a potential target for interventions to improve clinical outcome in CKD (Isakova *et al.,* 2009)

A significant elevation of phosphate has been recorded in ESRD patients than controls. Fourtounas

(2011) found that, in CKD, the kidneys fail to excrete the PO_4^{-2} and the result is positive PO_4^{-2} balance. The skeleton through the disorders of the bone that accompany CKD, contributes to this hyperphosphatemia, as it cannot handle the excessive phosphorus. Also, our data were in agreement with Komaba and Fukagawa (2009), who stated that, reduced renal function directly affects phosphorus re-absorption. The kidney is not capable of filtering enough phosphorus and its high level in blood directly stimulates the parathyroid gland which is the main organ responsible for Ca^{2+} homeostasis in the organism (Duran *et al.,* 2010). Extracellular ionic Ca^{2+} is the main parathyroid regulator, low levels stimulate PTH secretion and high levels inhibit hormone release, furthermore, favor its degradation within the parathyroid cells (Silver and Levi , 2005)

The present study also revealed significant increase in the PTH hormone levels but significant decrease in Ca^{2+} levels in ESRD patients than their control counterparts. These data were supported by Rodrinuez *et al.*(2006), who stated that in CKD the incorrect control of PTH secretion has attributed to the reduced vitamin D receptors and calcium receptor expression which occurs in parallel to the parathyroid gland growth. Parathyroid gland hyperplasia and the consequent increase in PTH secretion are responsible for hyperparathyroidism observed in CKD.

The hypocalcemia reported in this study was supported by the study of Levin *et al.* [2007] who reported that, in advanced cases of renal failure serum calcium levels drop in response to decreased intestinal calcium absorption. Moreover, Wesseling *et al.*[2008] stated that, hypocalcemia is quite uncommon in CKD stage 3 and early stage 4, but more often observed in stage 5.

In summary, our data suggest that immune cells in HD children are in an activated state which may explain the observed alterations in the levels of the assessed pro-inflammatory cytokines and adhesion molecules. In addition, significant negative associations between serum fetuin-A and serum inflammatory markers concentrations; sICAM-1, sVCAM-1, TNF-α, IL-1b and IL-23, were demonstrated. More clinical studies should, therefore, be performed to evaluate the impact of cytokine-lowering interventions on serum fetuin-A concentrations and VC in patients with ESRD.

It is also crucial for investigations to address the mechanisms and complications of inflammation that are manifested in pediatric patients with CKD in all stages, since early identification and intervention may generate the most efficient strategies for prevention and treatment of CVD in those patients.

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